IMMUNOHISTOCHEMICAL EXPRESSION AND EVALUATION OF MCM2 AND CYCLIN D1 IN ORAL SQUAMOUS CELL CARCINOMA AND VERRUCOUS CARCINOMA

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BRANCH – VI

ORAL PATHOLOGY AND MICROBIOLOGY

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ORAL PATHOLOGY AND MICROBIOLOGY

CERTIFICATE

This is to certify that **Dr. MENAKA.T.R** Post Graduate student (2016-2019) in the Department of Oral Pathology and Microbiology, Adhiparasakthi Dental College and Hospital, Melmaruvathur – 603319, has done this dissertation titled **"IMMUNOHISTOCHEMICAL EXPRESSION AND EVALUATION OF MCM2 AND CYCLIN D1 IN ORAL SQUAMOUS CELL CARCINOMA AND VERRUCOUS CARCINOMA"** under our direct guidance and supervision in partial fulfilment of the regulations laid down by **THE TAMILNADU DR.M.G.R MEDICAL UNIVERSITY**, Chennai – 600032 for **MASTER OF DENTAL SURGERY - (BRANCH-VI) ORAL PATHOLOGY AND MICROBIOLOGY** degree examination.

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DECLARATION

TITLE OF THE DISSERTATION	**IMMUNOHISTOCHEMICAL EXPRESSION AND EVALUATION OF MCM2 AND CYCLIN D1 IN ORAL SQUAMOUS CELL CARCINOMA AND VERRUCOUS CARCINOMA**
PLACE OF THE STUDY	ADHIPARASAKTHI DENTAL COLLEGE AND HOSPITAL, MELMARUVATHUR – 603319
DURATION OF THE COURSE	3 YEARS
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ABSTRACT

BACKGROUND:

Oral squamous cell carcinoma (OSCC), represents over 90% of malignancies of the oral cavity**.** Despite the advances in diagnosis and therapy, OSCC continues to have a shorter survival rate. Verrucous carcinoma (VC) of the oral cavity is a low grade variant of OSCC. The study of cell proliferation is important for assessing the tumor behaviour, prognosis and patient survival of both these tumours. As literature search did not reveal sufficient studies of immunohistochemical expression of Cyclin D1 and Mini Chromosome Maintainance 2 (MCM2) in OSCC and VC, the present study was done to evaluate the expression of these two cell proliferation biomarkers in Oral Squamous cell carcinoma and Verrucous carcinoma.

AIM:

To evaluate the immunohistochemical expression of MCM 2 and Cyclin D1 in oral squamous cell carcinoma and verrucous carcinoma.

MATERIALS AND METHODS:

This immunohistochemical study was conducted on the archives retrieved formalin fixed, paraffin embedded tissue sections from the Department of Oral and Maxillofacial Pathology, Adhiparasakthi Dental College and Hospital, Melmaruvathur. The study group included 20 cases of histopathologically diagnosed Oral Squamous Cell Carcinoma (10 cases of well differentiated squamous cell carcinoma, 10 cases of Moderately differentiated Squamous Cell Carcinoma) and 10 cases of histopathologically diagnosed verrucous carcinoma.

Control group included 10 biopsies from the normal buccal mucosa adjacent to the site of surgery during the surgical removal of third molars in patients. All samples were evaluated for the expression of Cyclin D1 and MCM 2 using standard immunohistochemistry procedure.

The present study involved both qualitative and quantitative analysis. Qualitative analysis was done by evaluation of intensity of staining and area of staining. Quantitative analysis was done by calculating the percentage of positively stained cells and assessing the Labelling Index. Data obtained was subjected to statistical analysis using SPSS statistical package (version 19.0).

RESULTS

On evaluating and comparing Cyclin D1 and MCM 2 intensity and area of staining between the groups, statistically significant values ($p<0.05$) were obtained using Kruskall Wallis' ANOVA. Comparison of LI of Cyclin D1 and MCM 2 in normal mucosa, OSCC and VC statistically significant results ($p<0.05$) were obtained using Mann Whitney U test. Mean LI of MCM2 was found to be significantly higher than mean LI of cyclin D1 in all the study groups.

CONCLUSION

From the present study we conclude that MCM2 has the potential to serve as a novel cell proliferation biomarker in OSCC and VC as compared to Cyclin D1.

Key Words: Oral squamous cell carcinoma, Verrucous carcinoma, Cyclin D1, MCM2, cell proliferation.

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LIST OF ABBREVIATIONS

OSCC	-	Oral Squamous Cell Carcinoma
HNSCC	-	Head and Neck Squamous cell carcinoma
HPV	-	Human Papilloma Virus
VC	-	Verrucous Carcinoma
SCC	-	Squamous Cell Carcinoma
NM	-	Normal Mucosa
DNA	-	Deoxyribonucleic Acid
CDK	-	Cyclin Dependent Kinase
Rb	-	Retinoblastoma
RNA	-	Ribonucleic Acid
MCM	-	Minichromosome Maintainance
G0	-	Resting Phase
G1	-	Gap 1 Phase
G2	-	Gap 2 Phase
S phase	-	Synthesis Phase
M phase	-	Mitosis Phase
AgNor	-	Argyrophilic Nucleolar Organiser Region
Kda	-	Kilodalton
CCND1	-	Cyclin D1 Protein coding gene
Bcl 1	-	B cell Lymphoma-1 protein
Cip/Kip	-	Cyclin Dependent Kinase Inhibitory protein
Cdc 6	-	Cell division cycle 6 protein
Cdt 1	-	Chromatin licensing and DNA replication factor 1

M APK	-	Mitogen Activated Protein Kinase
Wnt signalling	-	Wingless/ Integrated Signalling
M phase	-	Mitosis Phase
E2F	-	Transcription Factor
WDSCC	-	Well Differentiated Squamous Cell Carcinoma
MDSCC	-	Moderately Differentiated Squamous Cell Carcinoma
ORC	-	Origin Recognition Complex
Pre-RC	-	Pre- replication Complex
DSS	-	Disease Specific Survival Period
PVL	-	Proliferative Verrucous Leukoplakia
OTSCC	-	Oral Tongue Squamous Cell Carcinomas
LSCC	-	Laryngeal Squamous Cell Carcinomas
H & E	-	Hematoxylin and Eosin
GSK3β	-	Glycogen Synthase Kinase 3 beta
LI	-	Labelling Index
IHC	-	Immunohistochemistry
HRP	-	Horse radish peroxidase
DAB	-	Diamino benzidine
TBS	-	Tris buffered saline
H_20_2	-	Hydrogen peroxide
DPX	-	Distyrene Plasticizer Xylene
EDTA	-	Ethylene Diamine Tetra Acetic Acid
PCNA	-	Proliferating Cell Nuclear Antigen
NF-κβ	-	Nuclear Factor kappa-light chain enhancer of activated B cells

INTRODUCTION

Oral squamous cell carcinomas (OSCC) belonging to a larger subgroup of tumours termed head and neck squamous cell carcinomas (HNSCC) represents over 90% of malignant oral neoplasms[1]. According to the International Agency for Research on Cancer, the incidence rate of oral cancer in India is 12.6 per 100,000 people when compared to United States and the Middle East which is 10 per 1,00,000 and less than 2 per 1,00,000 respectively[2]. The high incidence of OSCC in India has been attributed to a variety of etiological factors such as smoking, smokeless tobacco chewing, alcohol consumption, spicy food intake and human papillomavirus (HPV) infections[1]. These factors may act individually or synergistically in oral carcinogenesis, a multistage process, that involves precancerous lesions, invasion and metastasis[3].

Verrucous carcinoma (VC), a rare tumor first described by Ackerman[4] is a low grade variant of OSCC and is being considered as a separate clinicopathologic entity distinct from OSCC because of its unique biologic behaviour and slow growing nature. VC has a limited propensity to metastasize, hence with a better prognosis than OSCC[5]. Few studies reveal that some foci of SCC may be observed in 20% of VC cases, making it a hybrid tumor and conferring a metastatic potential to it[6].

Numerous studies have been done with a prime objective of understanding the biology, diagnosis, prognosis, and management of OSCC and VC. Despite considerable advances in the diagnostic and therapeutic techniques, OSCC continues to present a poor prognosis with a two to five year survival rate of 50-60% depending upon the stage of disease and site affected[7]. Because the prognosis of the patients decrease with increasing tumor stage, it is of great importance to detect the tumor as early as possible. If OSCC is diagnosed at an early stage (T1N0) survival rate of upto 80% is noted but in the later stages(T3-T4), it falls to about 20–30%[8].

Recent studies have clarified that a variety of molecular events at cellular levels play extremely important roles in not only tumor development but also tumor progression[9]. Studies have supported that oral carcinogenesis emerge from the accumulation of genetic changes and epigenetic anomalies in the expression of multiple genes[10]. Consequently, special attention has turned towards molecular biomarkers which are biochemical components that can define molecular and cellular alterations in both normal cells and those associated with malignant transformation[11].

Several biomarkers have been used in order to provide additional information about these tumors, including the inactivation of tumor suppressor genes, angiogenesis, apoptosis and cell proliferation markers[11]. The molecular markers of interest are those involved in cell

cycle regulation of tumor cells since carcinoma is caused by uncontrolled proliferation of cells[12]. Assessment of cell proliferation activity in tumors has become a common tool used by histopathologists in order to provide useful information for diagnosis, clinical behavior, and therapy of tumours[13].

The commonly used cell proliferation markers in OSCC include Ki-67, PCNA (Proliferating Cell Nuclear Antigen), Geminin, Cyclin D1, and Cyclin B1[11]. Indeed, the strongest connection between Cyclins and oncogenesis have been reported in studies conducted in OSCC[14]. Recent studies have proposed that Minichromosome Maintainance (MCM) proteins could also be sensitive proliferation markers and serve as novel biomarkers to determine the diagnosis and prognosis of various premalignant and malignant lesions[15].

The orderly progression of the cells through the various phases of cell cycle is controlled by a series of proteins called “Cyclins,” which exert their effect by binding and activating the Cyclin-Dependent Kinases (CDK)[16]. Both CDKs and Cyclins are positive regulators of cell cycle[17]. Among the cyclins, Cyclin D1 appears to be important in the G1 phase which is the only phase where the extracellular stimuli like growth factors can have an effect on the cell cycle[16]. Amplification and overexpression of Cyclin D1 have been reported in head and neck, oral, laryngeal and nasopharyngeal carcinoma[18].

The minichromosome maintenance (MCM) proteins form a family of molecules that are essential in DNA synthesis and for the S-phase of cell cycle initiation [19]. MCM proteins form a heterohexameric ring of MCM2–MCM7 complexes that act as replicative DNA helicase [20] and ensure that DNA replicates only once per mitotic cycle [21].Because of its expression in the early G1 phase, few studies have demonstrated that MCM proteins can be used as proliferation markers for determing the tumour behaviour[20] and MCM-2 protein can be used not only to estimate the proliferative index, but also as a prognostic factor for the survival rate of patients with OSCC[11].

Therefore, detection of MCM2 and Cyclin D1 can be used to distinguish cells that exhibit aberrant cell proliferation activity promoting tumour cells toward more advanced stages causing invasion to deeper tissues and metastasis to regional lymph nodes[22,23] Though few studies have been carried out to detect the expression of Cyclin D1 and MCM2 in different grades of OSCC[24], literature search reveal very few studies on the expression of these markers in verrucous carcinoma. With this background, the present study has been undertaken to evaluate the immunohistochemical expression of MCM2 and cyclin D1 in well and moderately differentiated oral squamous cell carcinoma and verrucous carcinoma.

AIM & OBJECTIVES

Aim:

To evaluate the immunohistochemical expression of Cyclin D1 and MCM 2 in oral squamous cell carcinoma and verrucous carcinoma.

Objectives:

1. To determine and compare the intensities of expression of Cyclin D1 in oral Well differentiated squamous cell carcinoma, Moderately differentiated squamous cell carcinoma, Verrucous carcinoma and Normal mucosa
2. To determine and compare the area of staining of Cyclin D1 in oral Well differentiated squamous cell carcinoma, Moderately differentiated squamous cell carcinoma, Verrucous carcinoma and Normal mucosa
3. To determine and compare the intensities of expression of MCM 2 in oral Well differentiated squamous cell carcinoma, Moderately differentiated squamous cell carcinoma, Verrucous carcinoma and Normal mucosa
4. To determine and compare the area of staining of MCM 2 in oral Well differentiated squamous cell carcinoma, Moderately differentiated squamous cell carcinoma, Verrucous carcinoma and Normal mucosa

5. To compare the intensities of expression of Cyclin D1 and MCM2 in Well differentiated squamous cell carcinoma, Moderately differentiated squamous cell carcinoma and Verrucous carcinoma.
6. To compare the area of staining of Cyclin D1 and MCM2 in well differentiated squamous cell carcinoma, Moderately differentiated squamous cell carcinoma and Verrucous carcinoma.
7. To determine and compare the Labelling Index (LI) of Cyclin D1 in oral Squamous cell carcinoma, Verrucous carcinoma and Normal mucosa.
8. To determine and compare the Labelling Index (LI) of MCM 2 in oral Squamous cell carcinoma, Verrucous carcinoma and Normal mucosa.
9. To compare the LI of Cyclin D1 and MCM2 in oral Squamous cell carcinoma, Verrucous carcinoma and Normal mucosa.

REVIEW OF LITERATURE

GENERAL REVIEW

CELL PROLIFERATION MARKERS IN ORAL SQUAMOUS CELL CARCINOMA

Molecular alterations that cause abnormal biological behavior of cancer cells especially uncontrolled cell proliferation are based on aberrations of cell cycle regulation[11]. A normal cell cycle consists of resting phase (G0), followed by the interphase which starts with G1 (Gap 1) where the cell increases in size[25,26]. This is followed by the S-phase (synthesis) where the DNA replication occur. Then, the cell enters into G2 (Gap 2) phase which ensures that the cell is ready for mitosis (Figure 1). Mitotic phase (M phase) is composed of two major events, nuclear division (Karyokinesis) and cytoplasmic division (Cytokinesis) and daughter cells are formed.

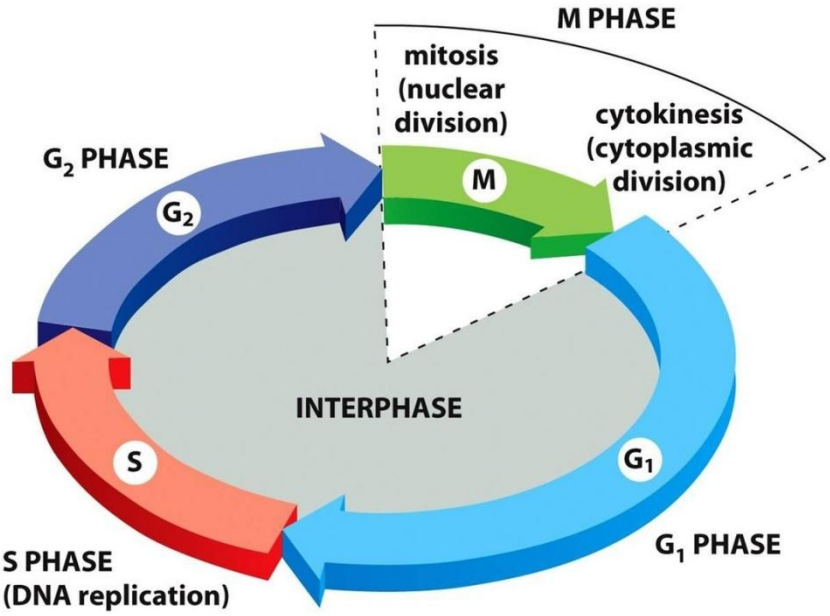


Figure 1: Cell Cycle showing interphase and mitosis

There are various check points present in the cell cycle, the important being G1 /S and G2/ M, both of these ensure that each phase is completed before the next one is initiated[16] . Restriction Point is no return point in the late G1 phase because once the cell crosses this, it is committed to another round of cell cycle[16]. Cell cycle progression is regulated by factors like Cyclins, CDKs, inhibitory enzymes, the retinoblastoma (Rb) protein, p21, p27 and p53[27]. After passing the restriction point late in G1, a cell will ignore exogenous signals and will enter DNA synthesis. After DNA synthesis, major intracellular insults, such as genomic damage or metabolic disruption can stop cell cycle progression, and the cells will be arrested at other checkpoints in S, G2, or M phase[11].

The study of cell proliferation is important for the assessment of tumor behavior, and this parameter has been linked with the assessment of prognosis, patient survival and tumor staging. Various techniques for the measurement of cell proliferation rate includes flow cytometry, which analyzes the percentage of cells in specific phases of the cell cycle, immunohistochemistry, genomics and proteomics. The cell proliferation markers can be grouped into three main categories[11]:

1. Growth fraction markers, such as Ki67
2. Cycle-specific markers (e.g., PCNA, cyclins)
3. Cell cycle time markers (e.g., AgNOR - Argyrophilic Nucleolar Organiser Region)

New cell proliferation markers are Minichromosome maintenance proteins (MCM) and geminin which helps in regulating cellular differentiation and proliferation. Both have been used as effective markers for early detection of cancer, especially in samples with suspected malignant epithelial tumor[28].

CYCLIN D1

Growth factors activates the regulatory proteins that control the transition through G1 phase of cell cycle[29]. Retinoblastoma (Rb) protein and E2F factor act as mediators of the G1 restriction point. Phosphorylation of Rb is carried out by cyclin/ cyclin-dependent kinase (CDK) complex which is a heterodimeric complex composed of a catalytic subunit, the CDK and a regulatory subunit called a cyclin[30].The CDKs are expressed constitutively during the cell cycle in an inactive form, and require cyclins, which are synthesized during specific phases of cell cycle to bind with them and activate them[16].

Over 30 Cyclin sequences have been identified and eight major classes of mammalian cyclins have been isolated and within some classes, a number of subclasses exist. So there are at least 11 cyclins which attain peak activity during the phases of cell cycle. CyclinD1-3, Cyclin E bind with CDK 4/6 and CDK 2 respectively, and regulate transition from G1 to S phase, whereas Cyclins A, B bind to CDK2 and CDK1 respectively and are most active during S and G2 phases where they regulate transition to the mitotic phase of the cell cycle (Figure 2)[30].

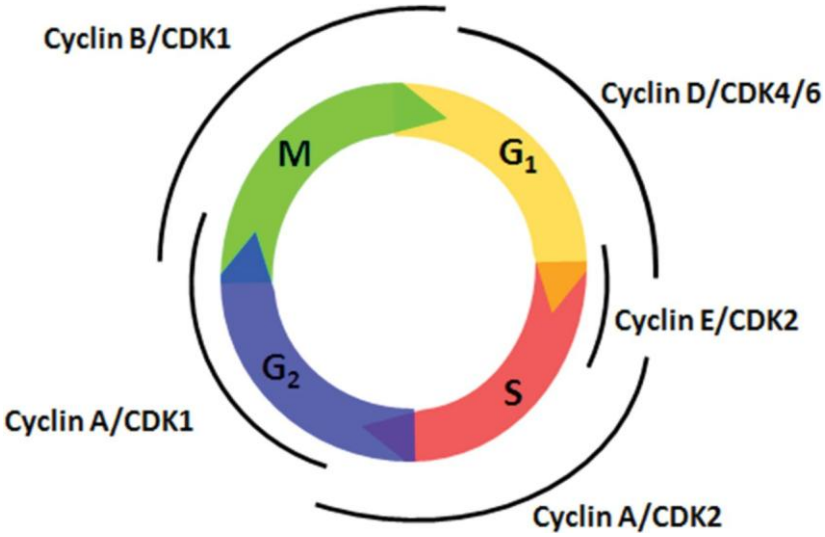


Figure 2: Cyclins and CDK substrates in various phases of cell cycle[31].

Among the cyclins, Cyclin D1, a 45 kDa (Kilodalton), 295 amino acid protein encoded by CCND1 gene located at chromosome 11q13 and reported with various other names like PRAD1 and Bcl-1 is an important cyclin in the G1-S transition[16]. Cyclin D1 was first isolated as PRAD1 oncogene clonally rearranged and overexpressed in parathyroid adenomas[17]. Cyclin D1 protein binds and activates CDK4 and CDK6 which leads to phosphorylation of retinoblastoma protein that results in the release of transcriptional activator E2F, leading to transcription and activation of proteins associated with passage through the G1 checkpoint thus facilitating progression into the S phase[16].

The D-type cyclin dependent kinase performs two functions crucial for passage through the G1 phase restriction point.

- The first function is catalytic and involves the phosphorylation-dependent inactivation of the Retinoblastoma protein (Rb). Rb phosphorylation results in the release of E2F complexes from Rb-dependent repression and the newly released E2F complexes are potent transcriptional activators of genes whose products regulate both the G1/S transition and S-phase such as cyclin E.
- The second function involves the incorporation of members of the Cip/Kip family of CDK inhibitory proteins into the cyclin D1/CDK4 complex and facilitates the activation of cyclin E/CDK2 complexes thereby promoting entry into the DNA synthetic phase of the cell division cycle.

The binding of Cip/Kip proteins facilitates both assembly of the cyclin D1 with CDK4 and ensure that nuclear localization occurs by inhibition of cyclin D1 nuclear export in the G1phase of the cell cycle[16,30].

Due to their crucial role in cell cycle regulation, D-type Cyclin have attracted considerable attention with regard to the involvement in oncogenesis. Overexpression of Cyclin D1 leads to shortening of the G1 phase and reduced dependency on growth factors[17], hence resulting in abnormal cell proliferation that in turn might favor the occurrence of

additional genetic lesions. This leads to disturbance in the normal cell cycle control and mitogenic signalling pathways increasing the cell transformation and tumourogenecity[9]. This overexpression can be detected by immunohistochemical staining by using anti–cyclin D1 antibody[17].

Over expression of Cyclin D1 is thought to provide the tumour cells with a selective growth advantage. Various factors responsible for overexpression of Cyclin D1 are:

- Gene amplification at 11q13.
- Chromosomal rearrangements and translocations (PRAD1, Bcl-1)
- Upregulation of gene transcription and post transcriptional mechanisms .
- Post translational stabilization of Cyclin D1 -GSK3β (Glycogen Synthase Kinase 3 beta)
- Retrovirus insertion .
- Alteration in synthesis or stability of Cyclin D1 protein [17].

MINI CHROMOSOME MAINTAINANCE-2 (MCM2)

Minichromosome Maintenance proteins (MCM) were first reported by Maine in 1984 in an attempt to identify factors that originate DNA replication[32]. Up to 10 MCM proteins have been discovered so far. MCM1 is a transcription factor; MCM2-7 proteins are best known among this family and are critical components of the replication initiation complex which initiates synthesis of DNA in eukaryotes. MCM10 is a ring shaped hexamer which physically links Helicase to DNA polymerase during DNA replication. MCM8 has a role in mitosis, while the role of MCM9 have not been elucidated completely. Several studies have shown that MCM proteins remain stable during cell cycle while their amount decreases significantly during differentiation. This is because the pre-replication complex is present throughout the cell cycle. This property makes these proteins suitable as proliferation markers[32].

Minichromosome maintenance proteins are essential factors for replication of DNA which were initially identified in Saccharomyces cerevisiae. Origin recognition complex (ORC) is a protein complex with the ability of binding to the origins of replication and forming a landing pad for the replication factors Cdc6 (Cell division cycle 6 protein) and Cdt1 (Chromatin licensing and DNA replication factor 1) (figure 3). At this time, MCMs (MCM2-7) are recruited to the chromatin. So, the pre-replication complex (pre-RC) is formed which allows S-phase to be initiated. After S-phase entry, this complex is

disassembled. MCM proteins and Cdc6 leave the chromatin following the increased activity of cyclin A-CDK2 (cyclin-dependent kinase2). Cdc6 is carried to the cytoplasm and Cdt1 is proteolysed. Any Cdt1 that has escaped proteolysis will bind to geminin[33]. The nuclear localization of the MCM 2-7 complex is regulated by the CDKs. So MCM 2-7 are imported into the nucleus when CDK activity is low in early G1 and exported from the nucleus during S phase when CDK activity is high[34]. As cells exit mitosis, these newly synthesized MCM proteins accumulate in the nucleus (early G1 phase) and assemble into pre-replicative complexes[34,35].

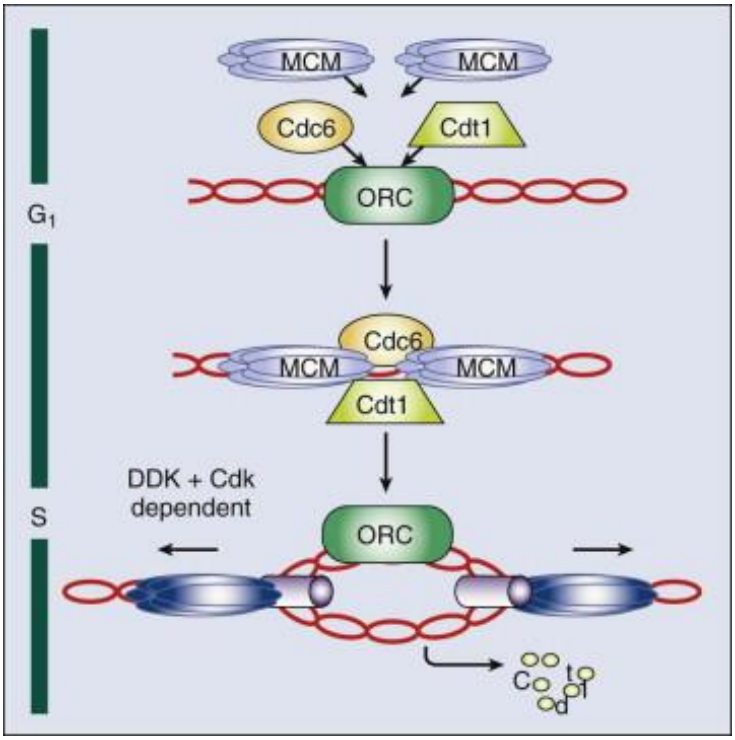


Figure 3- MCM proteins and ORC complex.Adapted from Marcos Malumbres, in Abeloff's Clinical Oncology (Fifth Edition),2014[35]

Minichromosome maintenance-2 (MCM-2) proteins regulates cell differentiation and cell proliferation. According to molecular studies, MCM-2 proteins identify both cycling cells and non-cycling cells with proliferative potential[36]. MCM2, forms a hexameric pre-replication complex (pre-RC) with other MCM proteins 3-7, and attaches to origin recognition complex (ORC) in association with Cdc6, which acts as a recruitment factor. MCM2 encodes a protein of 890 amino acids, and is homologous with MCM3[32].

When cells exit mitosis Mcm-2 accumulates in the nucleus (early G1 phase) and form the pre-replicative complexes with Cdc6,Cdt1, Cdc45 allowing CDK activated initiation of DNA synthesis during the subsequent phases thus licensing the cells to proliferate[32]. Antibodies against MCM-2 identify more cells in tissues in comparison with other proliferations markers such as Ki67. Many studies have shown overexpression of MCM-2 expression in carcinoma of kidney, colon, and larynx. Furthermore, recent studies conducted on precancerous and malignant lesions of oral cavity, and salivary gland tumours showed high expression of MCM-2[32].

CYCLIN D1 EXPRESSION IN ORAL SQUAMOUS CELL CARCINOMA AND VERRUCOUS CARCINOMA

Amplification of the CCND1 gene is the main oncogenic mechanism underlying cyclin D1 overexpression in OSCC but the alteration of pathways frequently involved in human carcinogenesis (MAPK, Wnt, NF-κβ) can also transcriptionally activate the CCND1 gene by increasing cyclin D1 expression[37,38]. These can cause the development of larger OSCCs and a higher risk of lymph node involvement[37].

- **Satoru Shintan *et al.,*(2001)[39]** evaluated the relationship between cyclin D1 expression levels and radiosensitivity in nine oral SCC cell lines and 41 clinical patients with oral SCC who underwent preoperative radiation therapy and found that the expression of cyclin D1 varied and the magnitude of the cyclin D1expression correlated with increased tumor radiosensitivity. Significant association between the response to preoperative radiation therapy and cyclin D1 overexpression was also observed in the oral SCC patients who were treated with preoperative radiation therapy. Their results suggested that cyclin D1 expression levels correlated with radiosensitivity and could be used to predict the effectiveness of radiation therapy on oral SCC.
- **J. Alan Diehl (2002)[40]** reviewed the role of cyclin D1 as a mitogenic sensor for the cell cycle machine and its role as cellular oncogene. He stated that Mitogen-dependent activation

of the cyclin D1 kinase occurs through increased transcription, protein accumulation, cyclin/CDK assembly, reduced cyclin proteolysis, and decreased nuclear export and the disturbances of which provides the cells with a distinctive growth advantage over their normal counterparts and represents an early event in neoplasia. The author also suggested that removal of proteins necessary for the proliferation of tumor cells through targeted degradation or subcellular sequestration or both in combination with kinase inhibitors might serve as an effective combinatorial therapy for certain malignancies.

- **Adriana da Costa Nevus *et al.*,(2004)**[41] correlated expression of cyclin D1 with the expression of p21 in 28 cases of oral squamous cell carcinoma (OSCC). No correlation was found between the mean numbers of cyclin D1 positive nuclei and p21 positive nuclei and the histological scores of malignancy. However, the marked expression of cyclin D1 in high-grade tumors supported its role in proliferative activity.
- **Masayuki Shiraki *et al.*, (2005)**[42] examined the impact of immunohistochemical expression of markers on tumor progression in 140 oral cancers and found that p53,cyclin D1 and EGFR (Epidermal Growth Factor Receptor) were expressed in 64 cases (46%), 54 cases (39%) and 54 cases (39%) respectively and thus concluded that simultaneous coexpression of these markers in oral cancers might prove to be a useful indicator for identification of low- or high-risk patients. Such

coexpression of multiple molecular markers is in close agreement with the fundamental concept of multistep cancer development and progression.

- **Punnya V. Angadi *et al* .,(2007)[17]** assessed the expression of cyclin D1 in 79 cases of OSCC and VC to compare its expression in both of these carcinomas, and found Cyclin D1 overexpression in 29 cases (70.7%) of OSCC and in 19 cases (63.3%) of VC. However no statistical significance was observed in cyclin D1 expression between OSCC and VC but statistical significance was seen between VC and poorly differentiated squamous cell carcinoma. The study concluded that the increased expression of cyclin D1 significantly correlated with lack of differentiation in malignant epithelial neoplasms since the results suggested a possibility that cyclin D1 targets not only proliferation but also affects the differentiation of the cells in these oral neoplasms[11].
- **John P Alao (2007)[43]** discussed the regulation of cyclin D1 degradation and the therapeutic ablation of cyclin D1 which might be useful for the prevention and treatment of cancer. He had stated in his article that in mammalian cells, DNA damage, environmental stress, genotoxic stress and viral infection have also been shown to induce the ubiquitin-dependent degradation of cyclin D1.

- **Jong Kyong Kim and J. Alan Diehl (2009)[44]** briefly provided overview of various mechanisms underlying aberrant cyclin D1 regulation in human cancers and their impact on neoplastic transformation. Their observations suggested that nuclear retention of active cyclin D1/CDK complex may be a critical determinant to elicit the oncogenicity of cyclin D1 in addition to its prevalent overexpression in cancers and its CDK-independent, nuclear receptor-agonistic activity may also contribute to its oncogenicity in certain types of cancers including breast cancer.
- **Satya N. Das *et al.*, (2011)[18]** investigated the correlation between cyclin D1 overexpression, clinicopathological features and cell cycle parameters in patients with tobacco-related OSCC. Higher expression of cyclin D1 was observed only in 30 of 45 cases that correlated with advanced age ($P < 0.02$), higher tumour stage ($P<0.01$), histological differentiation and lymph node metastasis ($P < 0.01$). Analysis of nuclear DNA pattern revealed cyclin D1 immunoreactivity in tumours with aggressive DNA pattern such as aneuploidy ($P<0.05$) and higher S phase fraction ($P<0.04$). The study concluded that higher expression of cyclin D1 in oral cancer appeared to be closely linked to cell proliferation, differentiation and lymph node invasion. Therefore pre-operative evaluation of cyclin D1 in biopsy specimen may be useful in planning the most appropriate treatment strategies in patients with tobacco-related OSCC.

- **Uma Swaminathan *et al.*, (2012)**[45] assessed p53 and cyclinD1 expression using immunohistochemistry in 20 cases of OSCC and 10 normal mucosa and stated that p53 and cyclin D1 is amplified and overexpressed in oral squamous cell carcinoma and a positive correlation is seen between increased mutant p53 expression and cyclin D1 expression in OSCC.
- **Shiang-Fu Huang *et al.*,(2012)**[46] examined cyclin D1 protein expression using immunohistochemistry in 264 males with OSCC and found overexpression of cyclin D1 in 97 cases of OSCCs which associated with lymph node metastasis, tumor cell differentiation and tumor stage. The study concluded that cyclin D1 protein worked as an independent prognostic factor and can be as a biomarker for the aggressiveness of OSCC.
- **Swati Saawarn *et al.*, (2012)**[47] immunohistochemically evaluated the expression of cyclin D1 in forty formalin-fixed paraffin-embedded tissue blocks of oral squamous cell carcinoma and found highest expression in well-differentiated, followed by moderately differentiated, and poorly differentiated squamous cell carcinomas, with a statistically significant correlation thus showing that Cyclin D1 expression significantly increases with increase in differentiation.
- **Lai-ping Zhong *et al.*,(2013)**[48] performed immunohistochemical staining for cyclin D1 in pretreatment biopsy specimens of 232 out of 256 clinical stage III/IVA OSCC patients randomized to the clinical trial. Cyclin D1 index was estimated as the

proportion of tumor cells with cyclin D1 nuclear staining. A low cyclin D1 expression predicted significantly better overall survival, disease-free survival, locoregional recurrence-free survival and distant metastasis-free survival compared to high cyclin D1 expression. The results concluded that cyclin D1 expression could be used as a biomarker in further validation studies to select patients who could benefit from induction therapy.

- **Yanhui Zhao *et al.*, (2014)[49]** performed a comprehensive meta-analysis for evaluation of cyclin D1 overexpression in oral squamous cell carcinoma to determine the strength of this association and concluded that cyclin D1 expression correlated with detrimental clinicopathological outcome and poor prognosis in oral squamous cell carcinoma. So The prognostic value of cyclin D1, either alone or combined with alternative molecular markers, would be validated in clinical practice.
- **Yuichi Ohnishi *et al.*, (2014)[50]** conducted an immunohistochemical investigation of cyclin D1 and Ki-67 expression in OSCC to evaluate the correlations between cell differentiation, cell proliferation and metastasis, the effect of anticancer drug medication and cyclin D1 expression. Cyclin D1 and Ki-67 were detected clearly in the nuclei of 35 SCC samples but no correlation was found between cyclin D1 protein expression and oral SCC differentiation. Their results indicated that the expression of cyclin D1 protein plays a role in cell

differentiation and cell proliferation in well-differentiated oral SCC and also the high expression of cyclin D1 may contribute to drug resistance in cancer cells, not only by increasing cell proliferation, but also by suppressing cancer cell apoptosis.

- **Swati Saawarn *et al.*, (2015)[16]** provided an insight into the physio-pathological role of cyclin D1 in Oral Squamous Cell Carcinoma and found that there is wide variation and diversity in the reports available in the literature regarding levels, diagnostic and prognostic significance of Cyclin D1 especially in oral Squamous cell carcinoma. The article also suggested that other than being used as a prognostic marker, cyclin D 1 is may also be used as a target molecule in cancer chemotherapy as tumors over-expressing cyclin D1 has been found to be more sensitive to chemotherapeutic agent Rapamycin.
- **Anand Choudhary *et al.*,(2016)[24]** performed an immunohisto chemical analysis in 50 cases of different grades of OSCC using anti-cyclin D1 antibody and found overexpression of cyclin D1 in 68% cases with a significant correlation with younger age group and also in the intergroup comparison of the cyclin D1 expression between well differentiated OSCC and poorly differentiated OSCC.
- **Reena Rachel John *et al.*, (2017)[51]** reviewed the multifaceted role played by cyclin D1 in various types of malignancies in different sites of the body and suggested that cyclin D1 is a promising tumor marker which can aid in the diagnosing and

predicting the prognosis of OSCC. They stated that a range of 35%–64% of head and neck squamous carcinomas shows overexpression of cyclin D1 and cyclin D1 - negative tumors reacted, especially well to multimodality treatment

- **Pablo Ramos-García *et al.*, (2017)**[38] reported the influence of CCND1/cyclin D1 on tumor size and clinical stage and provided an update on the utilization of cyclin D1 as therapeutic target and on the combination of cyclin D1 inhibitors with cytotoxic agents. He emphasised that cyclin D1 regulates cell migration and participates in metastatic development in OSCC and other tumours.
- **Sunit B. Patel *et al.*,(2017)**[52] compared cyclin D1 and p63 expression in leukoplakia and OSCC to investigate the possible correlation of both markers with grade of dysplasia and histological grade of OSCC. Their study concluded that the overall expression of cyclin D1 and p63 correlated with tumor differentiation and poor histological grades, from well-differentiated to poorly-differentiated SCC. Increased cyclin D1 and p63 expression was also associated with the severity of leukoplakia.
- **Pablo Ramos-García *et al.*, (2018)**[53] performed a systemic review and meta-analysis to evaluate the prognostic significance of cyclin D1 overexpression in OSCC and showed that cyclin D1 overexpression had a strong statistical association with worse overall survival, worse disease-free survival, higher T status, N+

status, advanced stage, and high histological grade. Their findings indicated that immunohistochemical assessment of cyclin D1 overexpression may be useful as a prognostic biomarker for OSCC and patients with tongue SCC, the most frequent intraoral site that carries the worst prognosis can especially benefit from the evaluation of cyclin D1 overexpression in their prognostic assessment. According to their findings, cases should be considered positive when the percentage of tumor cells with nuclear cyclin D1 expression is 10% or above.

MCM2 EXPRESSION IN ORAL SQUAMOUS CELL CARCINOMA AND VERRUCOUS CARCINOMA

- **Isamu Kodani *et al.*,(2001)[54]** examined cell cycle and cell death biomarker trends with the normal-dysplasia-carcinoma sequence of the oral epithelia analyzing the pathological significance of MCM2. The results showed significantly higher labeling indices of MCM2, Ki-67, and P53 in OSCCs than in the dysplasias and concluded that MCM2 regulated via a P53-independent pathway as a useful biomarker of proliferating cells.
- **IS Scott *et al.*, (2006)[55]** examined the potential utility of MCMs as candidate biomarkers for detecting oral malignancy and dysplasia in 101 smears of oral lesions. Of 52 conventional smears of SCC tissue samples, 18 were inadequate and MCM-positive cells were present in 33/34 adequate samples. They

concluded that MCMs are promising markers for early detection of oral SCC and dysplasia, particularly in a liquid-based cytology platform.

- **Jolanta Szelachowska *et al.*, (2006)[56]** evaluated the expression intensities of the MCM-2 protein and Ki-67 antigen in squamocellular carcinomas of the oral cavity in forty-nine patients, operated on and treated with radiotherapy and compared their prognostic value. A significant positive correlation was noted between the expression of MCM-2 protein and that of the Ki-67 antigen. The results suggested that the expression of MCM-2 protein may be used as a prognostic factor in patients with squamocellular carcinoma of the oral cavity.
- **A Torres-Rendon *et al.*,(2009)[28]** identified the the expression pattern of MCM2, Ki67 and geminin in normal oral mucosa ,oral epithelial dysplasia, and OSCC. MCM2 protein expression was found to be higher in the oral epithelial dysplasia with malignant progression and there was a significant increase in the MCM2/Ki67 and geminin/Ki67 ratios . The study concluded that MCM2 and geminin proteins as novel biomarkers of growth and hence useful prognostic tools for oral epithelial dysplasia.
- **Adriele Ferreira Gouveˆa *et al.*, (2010)[57]** evaluated the clinicopathological characteristics and the distribution of cell proliferation markers, p53, Ki-67, MCM-2 and MCM-5 aiming to elucidate the distinct biological behavior of the Proliferative verrucous leukoplakia (PVL) in 12 patients for whom 47

biopsies were taken which showed six cases of hyperkeratosis and acanthosis, 27 cases of mild dysplasia, three cases of moderate dysplasia, four cases of severe dysplasia and seven of SCC. The immunohistochemical findings showed higher positivity for p53, Ki-67, MCM-2 and MCM-5 in SCC and in some patients with mild or moderate dysplasia, specially the patients who developed SCC. The results suggested that high immunoexpression of MCM-2 and MCM-5 in mild and moderate dysplasia could be helpful to predict the malignant transformation of proliferative verrucous leukoplakia.

- **Giaginis C *et al.*,(2010)**[58] reviewed the the clinical significance of MCM protein expression in human neoplasia in comparison to conventional proliferative markers and revealed that MCM expression is associated with important clinicopathological parameters for patient management and also exhibited significant diagnostic and prognostic value in several malignancies. MCMs was characterized by higher specificity and sensitivity than the conventional proliferative markers, such as Ki-67 and PCNA, and thus was considered as diagnostic and prognostic tools of greater clinical significance in several types of human malignancy.
- **L. A. Gueiros *et al.*, (2011)**[59] evaluated the clinicopathological features and immunohistochemical expression of proliferation markers Ki-67, MCM-2 and geminin in sixty-three patients without previous treatment or distant metastases of oral tongue

squamous cell carcinomas (OTSCC). All markers showed a higher staining pattern on the periphery of the tumoural islands. MCM-2-positive cells were also present in the central portion of the tumours. MCM-2 presented higher LI followed by Ki-67 and geminin. MCM-2 immunoexpression was related to nodal recurrence, geminin was statistically associated with perineural invasion, Ki-67 presented a statistically significant association with distant metastasis and MCM-2 was statistically related to nodal recurrence and advanced disease stage. The results concluded that Anneroth and Bryne score in association with biomarkers of proliferation can be useful for evaluating the biological behaviour of OTSCC.

- **Heba N. Shalash *et al.*, (2012)[34]** conducted a study to examine the cellular distribution of MCM-2 in oral squamous cell carcinoma and their value to predict lymph node metastasis. Significant difference in the expression of MCM-2 between the different grades of OSCC cases and a positive correlation between the percentage of cases with positive lymph node metastasis and the mean area percentage of MCM-2 expression was noted, hence concluding that oral squamous cell carcinomas express MCM-2 with variable levels and cellular localization, making it an important marker of biological behavior in OSCC.
- **Motahhary P *et al.*, (2012)[60]** evaluated the expression of MCM2 and D2-40 in tongue squamous cell carcinoma (TSCC) and investigated their relation with lymph node metastasis and

patient survival. Lymphatic vessel density (LVD) was determined by D2-40 evaluation and MCM2 labelling index (LI) was also determined by counting of the positive cells. The result showed that the LVD of the group with lymph node metastasis was significantly higher than lymph node negative group and also showed a significant relation with patients survival but there was no significant relation between MCM2 LI and lymp node metastasis. Hence, the study concluded that D2-40 could be used as a marker in predicting nodal metastasis in TSCC.

- **Ramón Gil Carreón-Burciaga *et al.,*(2015)[61]** analyzed the presence of Ki-67 protein, MCM2 and MCM3 proteins in ameloblastoma and found that MCM2 and MCM3 showed higher proliferation indexes in ameloblastoma compared to Ki-67. Their results suggested MCM2 and MCM3 are more sensitive markers of cell proliferation.
- **Seyed Mohammad Razavi *et al.,*(2015)[36]** investigated the diagnostic value of MCM-2 expression in distinguishing histologically-proven normal oral mucosa, oral benign keratosis , oral epithelial dysplasia, and OSCC. Overexpression of MCM2 was noted with higher positivity in OSCCs and their findings indicated that MCM-2 could be a useful marker for early detection of OSCC and dysplasia.
- **Samar H. Zakaria *et al.*,(2016)[62]** assessed the cell proliferative activity of MCM-2 in oral epithelial dysplastic lesions and correlated the results with different grades of epithelial dysplasia

and found that MCM-2 immunostaining was found to increase gradually from mild to moderate to severe dysplasia and reached its maximum value in early invasive squamous cell carcinoma. The study concluded that MCM-2 has prognostic value in cases of oral dysplasia that have a tendency to undergo malignant transformation.

- **Kochli Channappa Niranjan *et al*.,(2018)[63]** assessed the expression of MCM-2 in Normal Oral Mucosa, Verrucous Hyperplasia , Verrucous Carcinoma and OSCC and compared it with the clinicopathological characteristics. There was a significant difference in MCM-2 expression with quantitative analysis among all the groups And there was a significant progressive increase in nuclear Labelling Indices from normal mocosa (49.08%), Verrucous Carcinoma (60.45%), Verrucous Hyperplasia with Dysplasia (64.10%) and OSCC (89.22%). Their findings suggested that MCM-2 may be useful for differentiating between Verrucous Hyperplasia with/ without dysplasia, Verrucous Carcinoma and OSCC.

CYCLIN D1 IN OTHER TUMOURS

- **Aleena Gladkikh *et al.*, (2010)[64]** determined the level of cyclin D1 expression in various B-cell lymphomas and observed that mantle cell lymphoma had the level of Cyclin D1 significantly elevated above that of normal lymphocytes.
- **Fereshteh Mohammadizadeh *et al.*,(2013)[65]** did a cross-sectional investigation in 89 patients with breast invasive ductal carcinoma and found reverse relationship between cyclin D1 overexpression and tumor grade
- **Yang Li *et al.*,(2014)[66]** observed that cyclin D1 overexpression was significantly associated with both poor overall survival and disease free survival in colorectal carcinoma.
- **Cheng-Han Lee *et al.*, (2012)[67]** identified diagnostic immunomarkers for biologically-defined Endometrial Stromal Sarcoma and observed upregulation of cyclin D1.
- **Somanath Padhi *et al.*, (2013)[68]** studied the pattern of cyclin D1 expression in 14 symptomatic patients of Multiple Myeloma and observed Cyclin D1 expression was observed in 8 of 14 cases.
- **R.A. Pereira *et al.*, (2014)[69]** assessed cyclin D1 expression in 85 patients who underwent radical prostatectomy for prostate carcinoma and observed high expression of cyclin D1 associated with a high-grade Gleason score (≤7) and the presence of perineural invasion.

MCM2 EXPRESSION IN OTHER TUMOURS

- **EJ Davidson *et al.*,(2003)[70]** investigated the expression of proliferation markers in Vulval intraepithelial neoplasia and detected Cyclins B1 and Cyclin D1 through the full thickness of the lesions.
- **Muhammad Zain Mehdi *et al.*, (2016)[71]** assessed the prognostic significance of MCM-2 and Ki-67 in renal cell carcinoma and found that Labeling index (LI) of MCM-2 was found to be much higher than Ki-67.
- **Katarzyna Nowinska *et al.*,(2016)[72]** studied the correlation between levels of expression of MCM proteins, Ki-67 proliferation antigen and metallothionein I/II (MT-I/II) in 83 laryngeal squamous cell cancer (LSCC) cases. Strong positive correlation was noted between expression of MCM2, MCM3, MCM7 and Ki-67 antigen in LSCC.
- **Bozena Werynska *et al.*,(2011)[73]** observed a positive correlation between expression of metallothionein I/II and expressions of Ki-67 and MCM-2 in non-small cell lung cancer.
- **H Gakiopoulou *et al.*, (2007)[74]** observed that the median MCM-2 and MCM-5 labelling indices (LIs) were significantly higher in adenocarcinomas and the levels of MCM-2 and MCM-5 increased significantly with advancing tumour stage.
- **Einas M Yousef *et al.*, (2017)[75]** studied the expression of MCM2 and Ki-67 in different histological grades and molecular subtypes of breast cancer and found that MCM2 and Ki-67 were highly expressed in breast tumors of high histological grades.

MATERIALS AND METHODS

MATERIALS

PARAFFIN BLOCKS:

Paraffin embedded tissues of histologically confirmed cases of Oral Squamous Cell Carcinoma, verrucous carcinoma and normal mucosa were used in the study.

EQUIPMENTS:

- Soft tissue microtome (Thermo scientific, MICROM HM340E)
- Paint brush
- Disposable microtome blades
- Hot plate
- Hot water bath
- Pathn Situ positively charged slides
- Pressure cooker (5 Liters)
- Measuring Jars
- Coplin Jars
- Electronic Timer
- Absorbent wipes
- Coverslip for slides
- Binocular Light Microscope (Olympus CX21i)
- Micropipette
- Micropipette tips
- Rectangular steel trough

- Tweezer
- Induction stove
- Incubator (Hitech Equipments)
- Liquid repellent slide marking pen
- Deparaffinization stainless steel staining trough and rack
- pH meter (E1 digital pH meter)
- A DELTA PLAN2 AP40 Trinocular Light Microscope with camera Head

ANTIBODIES:

1. Primary antibody

(a) Anti cyclin D1 [Rabbit monoclonal antibody] – EP12 (PathnSitu Biotechnologies Private Limited)

(b) Anti MCM 2 [Rabbit monoclonal antibody] – EP40 (PathnSitu Biotechnologies Private Limited)

2. Secondary kit (PolyExcel HRP/DAB Detection System) – Pathn Situ Biotechnologies Private Limited

a. PolyExcel H2 O2
b. PolyExcel Target Binder
c. PolyExcel Poly HRP
d. PolyExcel stunn DAB – Chromogen
e. PolyExcel stunn DAB – Buffer

REAGENTS:

- Tris-EDTA Buffer – 50X concentration (PathnSitu Biotechnologies Private Limited)
- Immuno wash Buffer – 25X concentration (PathnSitu Biotechnologies Private Limited)
- Distilled water
- Xylene
- Absolute alcohol (Isopropyl Alcohol)
- Alcohol 90% (Isopropyl Alcohol)
- Alcohol 70% (Isopropyl Alcohol)
- Harris Hematoxylin
- Mountant (Dibutyl Phthalate Xylene)

STUDY DESIGN AND PATIENT SELECTION:

This immunohistochemical study was conducted on the archival retrieved from the formalin fixed, paraffin embedded tissues retrieved from the archives of the Department of Oral and Maxillofacial Pathology, Adhiparasakthi Dental College and Hospital, Melmaruvathur. The study group included 20 cases of histopathologically diagnosed Oral Squamous Cell Carcinoma (10 cases of well differentiated squamous cell carcinoma, 10 cases of Moderately differentiated Squamous Cell Carcinoma) and 10 cases of histopathologically diagnosed verrucous carcinoma. Control group included 10 biopsies from the normal buccal mucosa adjacent to the site of surgery during the surgical removal of third molars in patients.

For positive control, archival retrieved formalin fixed, paraffin embedded tonsil tissue was obtained from the Department of General Pathology, Melmaruvathur Adhiparasakthi Institute of Medical Science and Research, Melmaruvathur.

IHC METHODOLOGY:

- Formalin fixed paraffin embedded tissues were sectioned at 3µm and mounted on positively charged slides. The slides were kept for incubation at 37^{o}C overnight.
- The slides were deparaffinized by 2 changes of xylene for a duration of 10 minutes each.
- The slides were hydrated through descending grades of alcohols as follows:
    - Absolute alcohol – 1 change, 5 minutes
    - 90% alcohol – 5 minutes
    - 70% alcohol – 5 minutes
- The slides were washed in distilled water (2 changes, 5 minutes each).
- Antigen retrieval was done using Tris EDTA (Ethylene Diamine Tetra Acetic acid) buffer by pressure cooker method (15- 20 minutes, upto 2 whistles) and cooled for about 30 minutes.
- The slides were washed in distilled water (2 changes, 3 minutes each).
- Using liquid repellent pen, circles were marked enclosing the section.

- PolyExcel H_2O_2 (Hydrogen Peroxide) was added on the section, kept for 10 minutes and endogenous peroxidase blocking was done.
- The slides were washed in wash buffer(5 minutes, 2 changes)
- Primary antibody was added and kept for 45 minutes for cyclin D1 and MCM2 in a moist chamber
- The slides were washed in wash buffer(5 minutes, 2 changes)
- PolyExcel Target Binder reagent was added and kept for incubation for 12 minutes
- The slides were washed in wash buffer(5 minutes, 2 changes)
- Polyexcel HRP(Horse Radish Peroxidase) was added and incubated for 12 minutes
- DAB(Diamino benzidine) solution was prepared (1 ml of DAB buffer + 1 drop DAB chromogen and mixed well)
- The slides were washed in wash buffer(5 minutes, 3 changes)
- Working DAB chromogen was added, kept for 5 minutes and then washed in distilled water.
- The slides were counterstained with Harris hematoxylin for 30 seconds and washed in running tap water for 5 minutes
- The slides were dehydrated through successive changes of alcohol, cleared with xylene, dried and mounted with DPX (Distyrene Plasticizer Xylene).

POSITIVE CONTROLS

Positive control section included tonsil for Cyclin D1(Figure 8) and MCM 2 (Figure 22)and was treated in the same manner as the test groups.

NEGATIVE CONTROLS

One section of test sample was selected and treated in the same manner as the test groups except that, the primary antibody was omitted for both Cyclin D1 and MCM 2.

ANALYSIS OF AREA OF STAINING OF CYCLIN D1 AND MCM 2

To know the expression pattern and also to determine the levels of protein expression in the epithelial layers, area of staining was analysed. It was determined by scanning the entire section of the epithelium at 4 X magnification and area of stained epithelial cells is recorded as:

SCORE	INFERENCES
0	0%
1	<25%
2	25 - 49%
3	50 - 74%
4	75 - 100%

The staining intensity was calculated for Cyclin D1 and MCM2 in WDSCC, MDSCC, VC and NM.

ANALYSIS OF STAINING INTENSITY OF CYCLIN D1 AND MCM2 :

To know the extent of stain uptake, intensity of staining was analysed. Ten random fields were selected at 40 X magnifications in each slide. Sections were scored for staining intensity and scaled as follows[17,23,24]:

SCORE	INFERENCES
0	No stain
1	Mild staining
2	Moderate staining
3	Intense staining

The staining intensity was calculated for Cyclin D1 and MCM2 in WDSCC, MDSCC, VC and NM.

ANALYSIS OF LABELLING INDEX (LI) OF CYCLIN D1 AND MCM 2 :

The slides were examined under a light microscope (Olympus CX21 i) at 40 X magnification and representative photomicrographs were taken in five hotspot areas for each slide. The photomicrographs were then analysed using image processing program (ImageJ, http://imagej.nih.gov/ij/). Percentage of IHC positive tumour cells per hot spot (A) was calculated and total number of tumour cells in each slide was calculated till a minimum of 400 cells were reached i.e the sum of the denominators (x)[24].

Percentage of positive nuclei (Labeling index-LI) in each case was calculated using the formula[34]

$$LI\ \% = \frac{A}{\text{Total no. of tumor cells}(x)} \times 100$$

The Labelling Index (LI) was calculated for Cyclin D1 and MCM2 in WDSCC, MDSCC, VC and NM.

Figure 4: Primary and Secondary Antibody kit

Figure 4a : Primary antibody Anti – Cyclin D1 [Rabbit Monoclonal]

Figure 4b : Primary antibody Anti – MCM 2 [Rabbit Monoclonal]

Figure 4c : Secondary Antibody kit [H2O2, Target Binder, Poly HRP]

Figure 4d : DAB Chromogen and DAB buffer

Figure 5: Reagents

Figure 5a : Tris- EDTA Buffer

Figure 5b : Hematoxylin

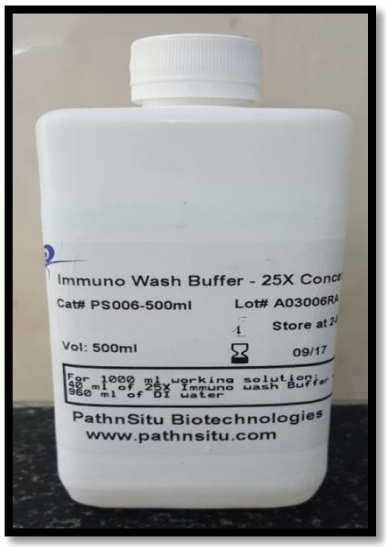


Figure 5c : Wash Buffer

Figure 6: Equipments

Figure 6a : Deparaffinization stainless steel staining trough and rack

Figure 6b : Micropipette

Figure 6c : Incubator

Figure 6d : Reagent Blocker Pen

Figure 6e : Microtome

Figure 6f : Microscope

Figure 6g : pH Meter

7. Determination of Labelling Index

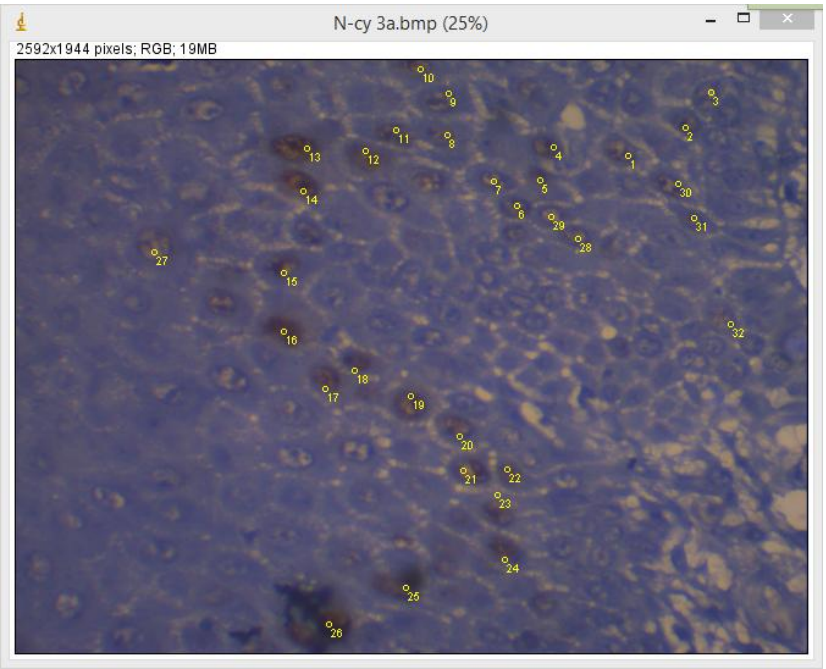


Figure 7a: Photomicrograph showing counting of IHC positive tumour cells using ImageJ software

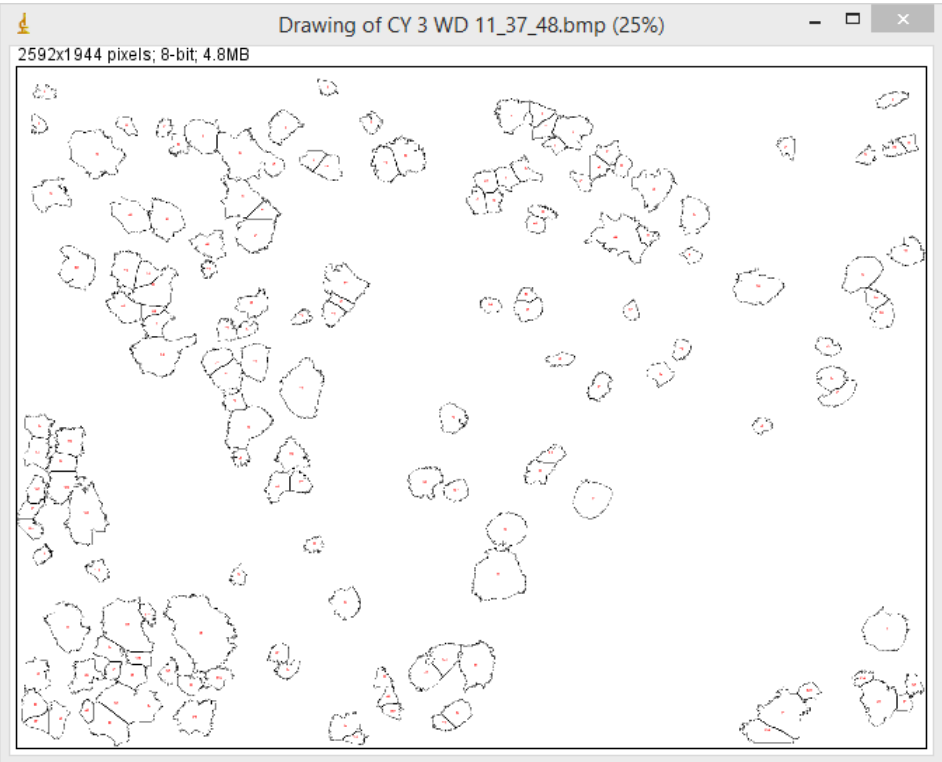


Figure 7b: Photomicrograph showing counting of total number of tumour cells in the field using ImageJ software

RESULTS

A retrospective cross sectional immunohistochemistry study was carried out on 40 archival retrieved formalin fixed paraffin embedded tissue blocks divided into 4 groups comprising of 20 histopathologically diagnosed cases of OSCC, 10 cases of Verrucous Carcinoma and 10 cases of normal mucosa (NM) to study the expression of CyclinD1 and MCM2, cell proliferation biomarkers. 20 OSCC cases were further divided into 2 subgroups; Well differentiated SCC (n=10) and Moderately differentiated SCC (n=10).

The presence of brown coloured end product at the site of target antigen indicated positive staining. All the cases showed variable intensities of nuclear staining. The present study involved both qualitative and quantitative analysis. Qualitative analysis was done by evaluation of intensity of staining and area of staining.

For assessing the intensity of staining 10 random fields were selected under 40x magnification and scored as 0- no stain, 1- mild stain, 2-moderate stain and 3-intense stain. For assessing the area of staining the entire section of the epithelium was scanned and area of stained epithelial cells were recorded as 0- 0%, 1- <25%, 2- 25 to 49%, 3- 50 to 74%, 4- 75 to 100%.

Quantitative analysis was done by calculating the percentage of positively stained cells. Representative fields were selected in each case and up a minimum of 400 tumor cells were counted and the percentage (%) of their positivity was determined. The % of positivity was denoted as labelling index (LI).

1.Determination and comparison of the intensity of expression and area of staining of Cyclin D1 in NM, WDSCC, MDSCC and VC

Cyclin D1 positivity was seen in all cases except one case of MDSCC. The observations of staining intensity and area of staining of Cyclin D1 in the present study are tabulated in Table 1 and Table 2 respectively.

Table 1: Staining intensity of Cyclin D1 among the study groups

SAMPLE	n	CYCLIN D1 -INTENSITY OF STAINING			
		NO STAIN	MILD	MODERATE	INTENSE
NM	10	0	6	3	1
WDSCC	10	0	0	2	8
MDSCC	10	1	2	2	5
VC	10	0	3	3	4

Table 2: Area of Staining of Cyclin D1 among the study groups

SAMPLE	n	CYCLIN D1 -AREA OF STAINING				
		SCORE 0	SCORE 1	SCORE 2	SCORE 3	SCORE 4
NM	10	0	6	3	1	0
WDSCC	10	0	0	2	4	4
MDSCC	10	1	6	1	1	1
VC	10	0	6	2	2	0

The mean score for intensity of staining for NM, WDSCC, MDSCC and VC were found to be 1.5 (SD 0.7), 2.8 (SD 0.4), 2.1 (SD 1.1) and 2.1 (SD 0.8) respectively. The difference between the mean scores was found to be statistically significant ($p<0.05$) using Kruskall Wallis' ANOVA (Table 3) (Graph 1).

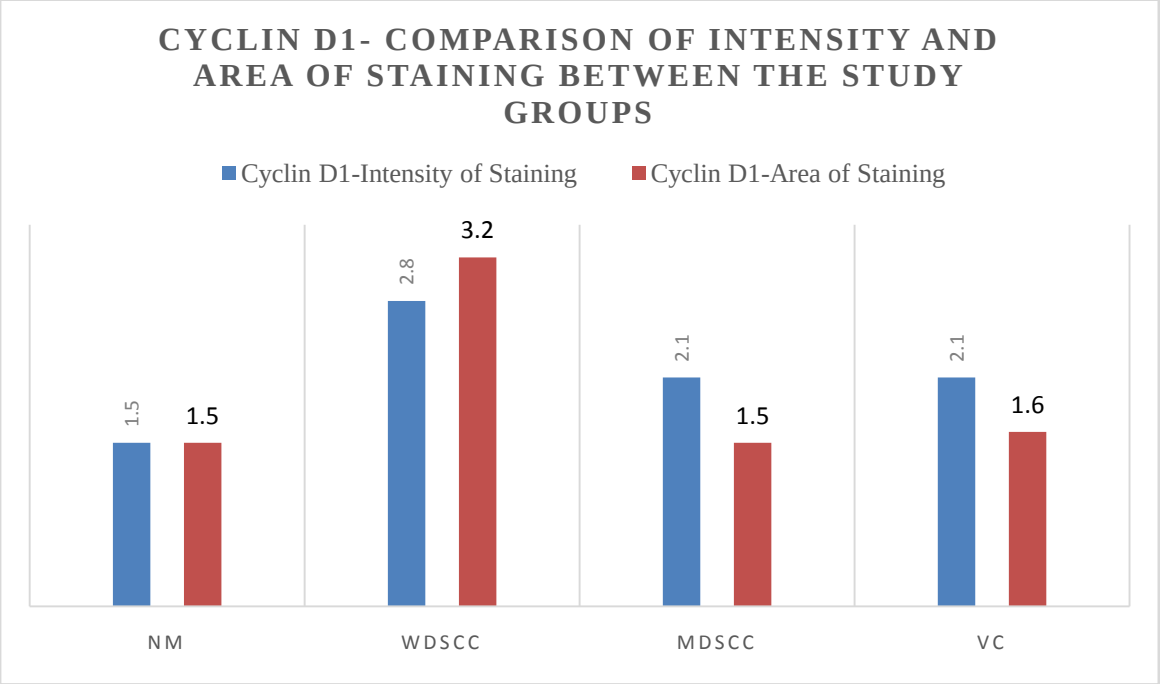
The mean score for area of staining for NM, WDSCC, MDSCC and VC were found to be 1.5 (SD 0.7), 3.2 (SD 0.7), 1.5 (SD 1.1) and 1.6 (SD 0.8) respectively. The difference between the mean scores was found to be statistically significant ($p<0.05$) using Kruskall Wallis' ANOVA (Table 3) (Graph 1).

TABLE 3: Comparison of Cyclin D1 intensity and area of staining between the study groups

		n	Mean	Std. Deviation	Mean Rank	Chi-Square	P-value
CyclinD1-Intensity of staining	NM	10	1.500	0.707	12.60	10.897	0.012*
	WDSCC	10	2.800	0.422	28.70		
	MDSCC	10	2.100	1.101	20.75		
	VC	10	2.100	0.876	19.95		
Cyclin D1-Area of Staining	NM	10	1.500	0.707	16.50	15.864	0.001*
	WDSCC	10	3.200	0.789	32.50		
	MDSCC	10	1.500	1.179	15.70		
	VC	10	1.600	0.843	17.30		

* denotes statistically significant using Kruskall Wallis' ANOVA

Graph 1: Comparison of Cyclin D1 intensity and area of staining between the study groups



2.Determination and comparison of the intensity of expression and area of staining of MCM 2 in NM,WDSCC, MDSCC and VC.

MCM2 positivity was seen in all cases. The observations of staining intensity and area of staining of MCM 2 in the present study are tabulated in Table 4 and Table 5 respectively.

Table 4: Staining intensity of MCM2 among the study groups

SAMPLE	n	MCM2 -INTENSITY OF STAINING			
		NO STAIN	MILD	MODERATE	INTENSE
NM	10	0	2	5	3
WDSCC	10	0	0	0	10
MDSCC	10	0	2	0	8
VC	10	0	1	2	7

Table 5: Area of Staining of MCM2 among the study groups

SAMPLE	n	MCM2 -AREA OF STAINING				
		SCORE 0	SCORE 1	SCORE 2	SCORE 3	SCORE 4
NM	10	0	4	5	1	0
WDSCC	10	0	0	1	2	7
MDSCC	10	0	2	2	1	5
VC	10	0	3	2	3	2

The mean score for intensity of staining for NM, WDSCC, MDSCC and VC were found to be 2.1 (SD 0.7), 3.0 (SD 0.0), 2.6 (SD 0.8) and 2.6 (SD 0.6) respectively. The difference between the mean scores was found to be statistically significant ($p<0.05$) using Kruskall Wallis' ANOVA (Table 6) (Graph 2).

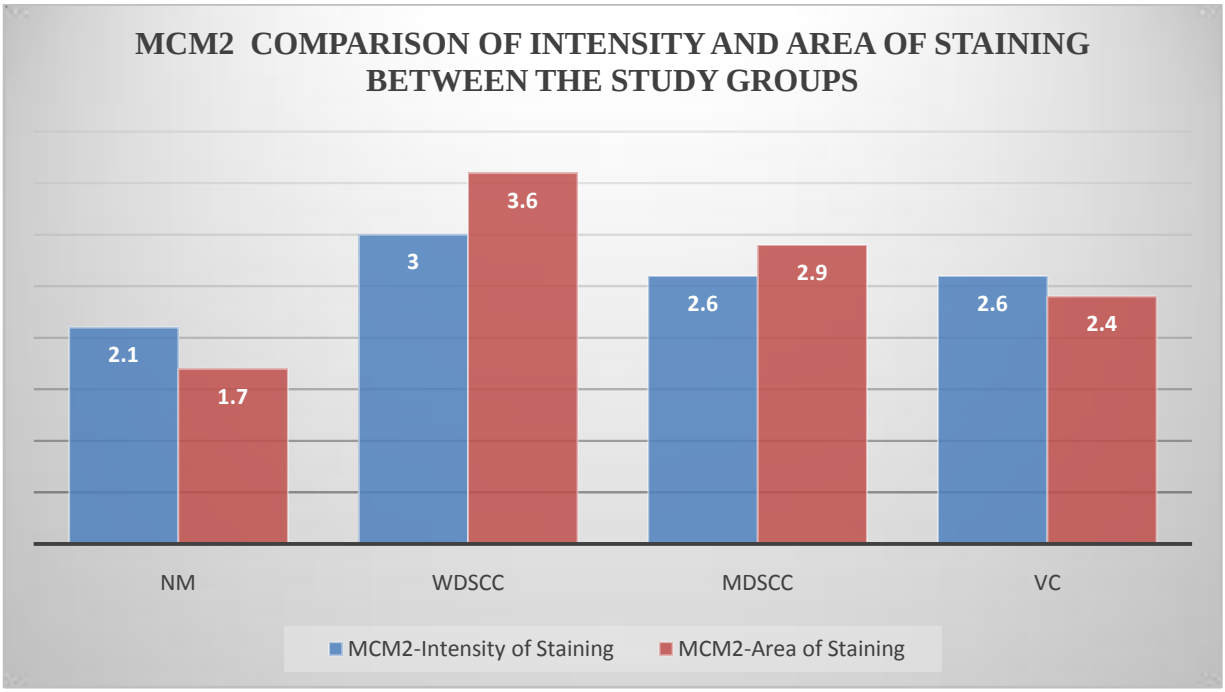
The mean score for area of staining for NM, WDSCC, MDSCC and VC were found to be 1.7 (SD 0.6), 3.6 (SD 0.6), 2.9 (SD 1.2) and 2.4 (SD 1.1) respectively. The difference between the mean scores was found to be statistically significant ($p<0.05$) using Kruskall Wallis' ANOVA (Table 6) (Graph 2).

TABLE 6: Comparison of MCM 2 intensity and area of staining between the study groups

		n	Mean	Std. deviation	Mean Rank	Chi-Square	P-value
MCM2-Intensity of Staining	NM	10	2.100	0.738	13.05	10.492	0.015*
	WDSCC	10	3.000	0.000	26.50		
	MDSCC	10	2.600	0.843	21.80		
	VC	10	2.600	0.699	20.65		
MCM2-Area of Staining	NM	10	1.700	0.675	11.55	13.708	0.003*
	WDSCC	10	3.600	0.699	29.50		
	MDSCC	10	2.900	1.287	22.95		
	VC	10	2.400	1.174	18.00		

* denotes statistically significant using Kruskall Wallis' ANOVA

Graph 2: Comparison of MCM 2 intensity and area of staining between the study groups



3. Comparison of staining intensities and area of staining between Cyclin D1 and MCM2 in WDSCC, MDSCC and VC

On comparing the intensities of expression of staining between Cyclin D1 and MCM2 in well differentiated squamous cell carcinoma, statistically insignificant value ($p > 0.05$) was obtained by using Mann Whitney U test. This could be because both markers predominantly showed intense staining (score 3) in WDSCC (Table 7) (Graph 3).

Similarly on comparing the area of staining between Cyclin D1 and MCM2 in well differentiated squamous cell carcinoma, statistically insignificant value ($p > 0.05$) was obtained by using Mann Whitney U test. This could be because both markers predominantly showed 74-100 % positive cells (score 4) in WDSCC (Table 7) (Graph 3).

On comparing the intensities of expression of staining between Cyclin D1 and MCM2 in MDSCC, statistically insignificant value ($p > 0.05$) was obtained by using Mann Whitney U test. (Table 7) (Graph 3). This could be because both markers predominantly showed intense staining (score 3) in MDSCC.

Similarly on comparing the area of staining between Cyclin D1 and MCM2 in MDSCC, statistically significant value ($p < 0.05$) was obtained by using Mann Whitney U test (Table 7) (Graph 3).

Though Cyclin D1 predominantly showed moderate to intense staining (score 2 and 3) and MCM 2 predominantly showed intense staining (score 3), on comparing the intensities of expression of staining between Cyclin D1 and MCM2 in VC, statistically insignificant value (p> 0.05) was obtained by using Mann Whitney U test (Table 7) (Graph 3).

Similarly on comparing the area of staining between Cyclin D1 and MCM2 in VC, statistically insignificant value (p> 0.05) was obtained by using Mann Whitney U test. This can be because in VC, Cyclin D1 showed mostly < 24% positive cells (score 1) whereas MCM2 showed both < 24% and 74-100 % positive cells (score 1 and 4) (Table 7) (Graph 3).

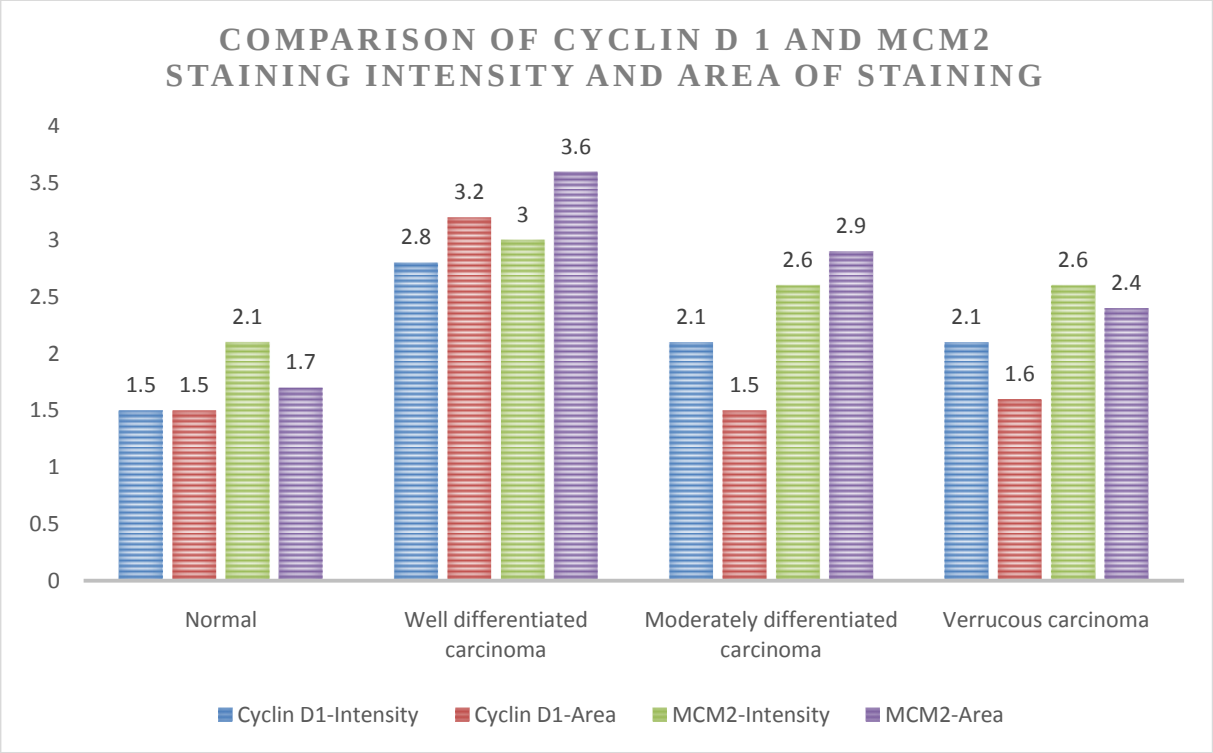
TABLE 7: Comparison between Cyclin D1 and MCM2 staining intensity and area of staining in WDSCC, MDSCC and VC

Comparison		WDSCC			MDSCC			VC		
	n	Mean	STD	P-Value	Mean	STD	P-Value	Mean	STD	P-value
Cyclin D1- I	10	2.8.	0.422	0.157	2.1	1.101	0.059	2.1	0.876	0.248
MCM 2 - I	10	3	0		2.6	0.843		2.6	0.699	
Cyclin D1- A	10	3.2	0.789	0.102	1.5	1.179	0.011*	1.6	0.843	0.066
MCM2 -A	10	3.6	0.699		2.9	1.287		2.4	1.174	

* denotes statistically significant using Mann Whitney U test

I – Staining Intensity A – Area of Staining

Graph 3: Comparison between Cyclin D1 and MCM2 staining intensity and area of staining in WDSCC, MDSCC and VC



4. Comparison of Labelling Index (LI) of Cyclin D1 among the study groups

The LI of cyclin D1 in Normal buccal mucosa ranged from 2.48%-17.03% with the mean score of 8.42 (SD-5.10). The LI of cyclin D1 in OSCC ranged from 11.78% - 30% with the mean value of 20.086 (SD-6.840). The LI of cyclin D1 in VC ranged from 9.34% - 44.48% with the mean score of 21.68 (SD-10.90).

On comparison of Labelling Index (LI) of cyclin D1 between the groups, statistically significant value ($p<0.05$) was obtained (Table 8) using Kruskall Wallis' ANOVA.

TABLE 8 : Comparison of Labelling Index (LI) of Cyclin D1 between the study groups

		n	Mean	Std. Deviation	Mean ranks	Chi-square	P-value
Cyclin D1 LI	NM	10	8.410	5.098	8.3	14.545	0.001*
	OSCC	20	20.086	6.840	24.8		
	VC	10	21.681	10.901	24.1		

* denotes statistically significant using Kruskall Wallis' ANOVA

5. Comparison of Labelling Index (LI) of MCM 2 among the study groups

The LI of MCM 2 in Normal buccal mucosa ranged from 12.58% - 22.56%. The mean score was 18.33 (SD-3.99). The LI of MCM 2 in OSCC ranged from 15.39% - 69.66% with the mean value of 43.599 (SD-15.330). The LI of MCM 2 in VC ranged from 10.08% - 64.43% with the mean value of 40.67 (SD-19.84).

On comparison of Labelling Index (LI) of MCM 2 between the study groups, statistically significant value ($p<0.05$) was obtained (Table 9) using Kruskall Wallis' ANOVA.

TABLE 9 : Comparison of Labelling Index (LI) of MCM 2 among the study groups

		n	Mean	Std. Deviation	Mean ranks	Chi-square	P-value
MCM2 LI	NM	10	18.331	3.993	8.4	14.451	0.001*
	OSCC	20	43.599	15.330	25.15		
	VC	10	40.667	19.836	23.3		

* denotes statistically significant using Kruskall Wallis' ANOVA

6. Comparison of Labelling Index (LI) between Cyclin D1 and MCM2 in NM, OSCC and VC

On comparing the Labelling Index (LI) between Cyclin D1 and MCM2 in normal mucosa, statistically significant valve was obtained ($p < 0.05$) using Mann Whitney U test (Table 10) (Graph 4).

On comparing the Labelling Index (LI) between Cyclin D1 and MCM2 in OSCC, statistically significant valve was obtained ($p < 0.05$) using Mann Whitney U test (Table 10) (Graph 4).

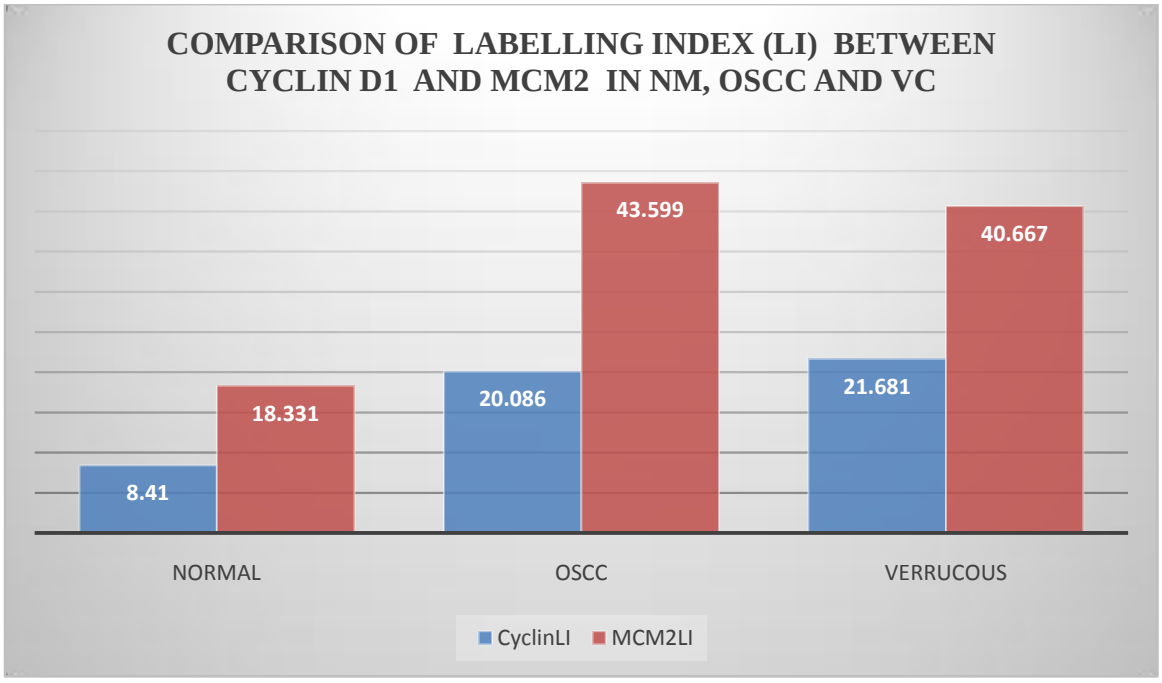
On comparing the Labelling Index (LI) between Cyclin D1 and MCM2 in Verrucous Carcinoma, statistically significant valve was obtained ($p < 0.05$) using Mann Whitney U test (Table 10) (Graph 4).

TABLE 10 : Comparison of Labelling Index (LI) between Cyclin D1 and MCM2 in NM, OSCC and VC

Comparison	NM (n=10)			OSCC (n=20)			VC (n=10)		
	Mean	STD	P-value	Mean	STD	P-value	Mean	STD	P-Value
Cyclin D1 LI	8.41	5.098		20.086	6.840		21.681	10.902	
MCM2 LI	18.331	3.993	0.007*	43.599	15.330	0.001*	40.667	19.836	0.017*

* denotes statistically significant using Mann Whitney U test

Graph 4: Comparison of Labelling Index (LI) between Cyclin D1 and MCM2 in NM, OSCC and VC



IMAGES –CYCLIN D1

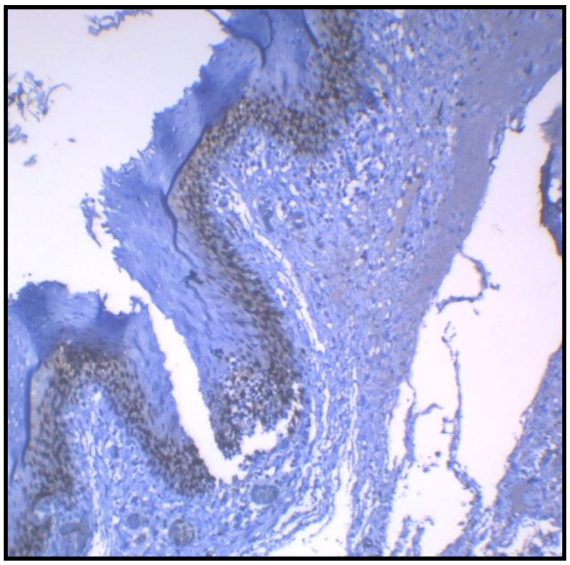


FIGURE 8: cyclin D1 expression in positive control Tonsil at 10 X magnification

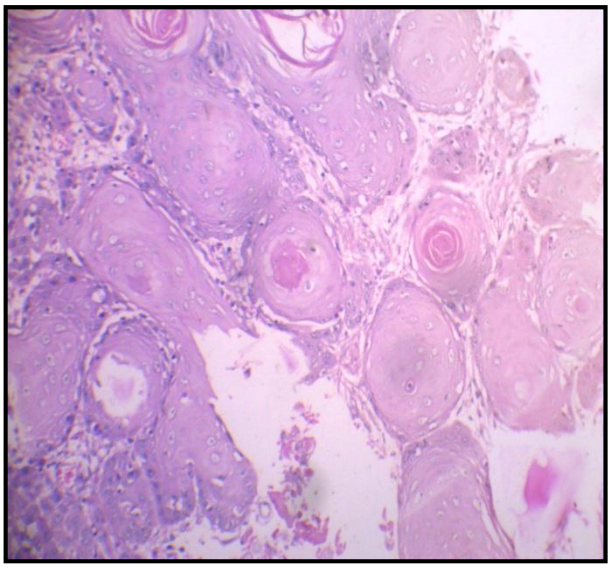


FIGURE 9: Photomicrograph showing H & E stained section of WDSCC at 10 X magnification

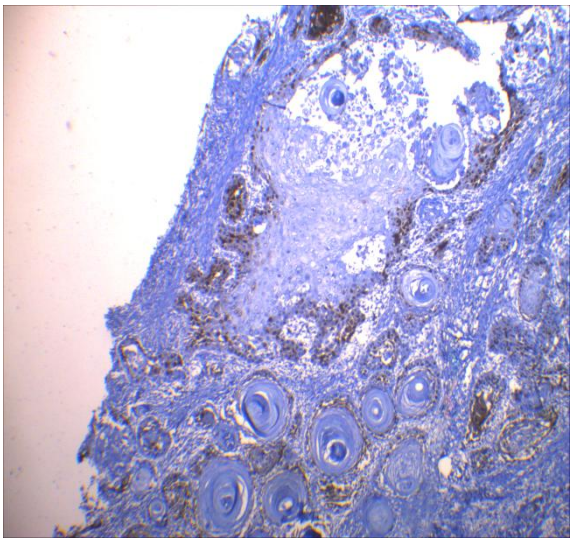


FIGURE 10: Cyclin D1 expression in WDSCC with more than 75 % of area of staining (score 4) at 4 X magnification

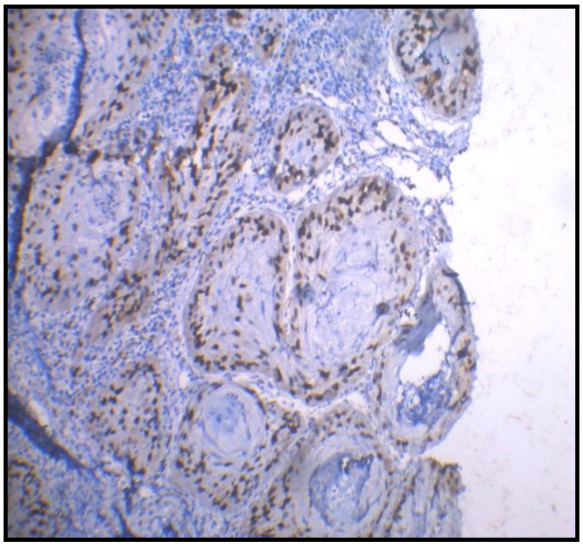


FIGURE 11: Cyclin D1 expression in WDSCC with 50- 74 % of area of staining (score 3) at 10 X magnification

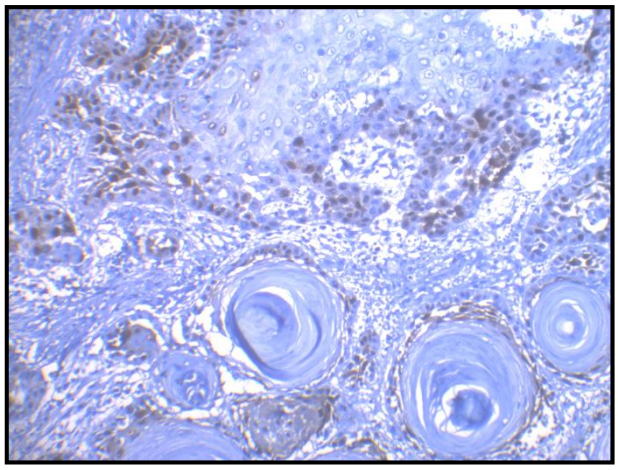


FIGURE 12: Cyclin D1 expression in WDSCC with intense staining (score 3) at 10 X magnification

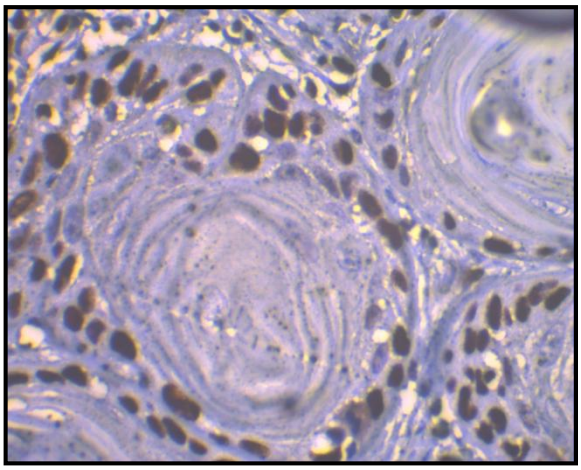


FIGURE 13: Cyclin D1 expression in WDSCC with intense staining (score 3) at 40 X magnification

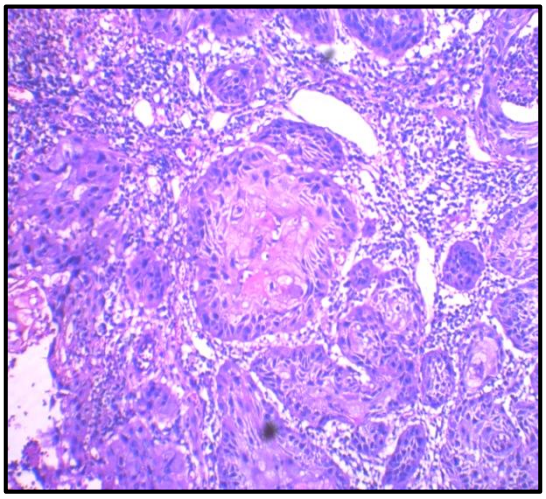


FIGURE 14: Photomicrograph showing H & E stained section of MDSCC at 10 X magnification

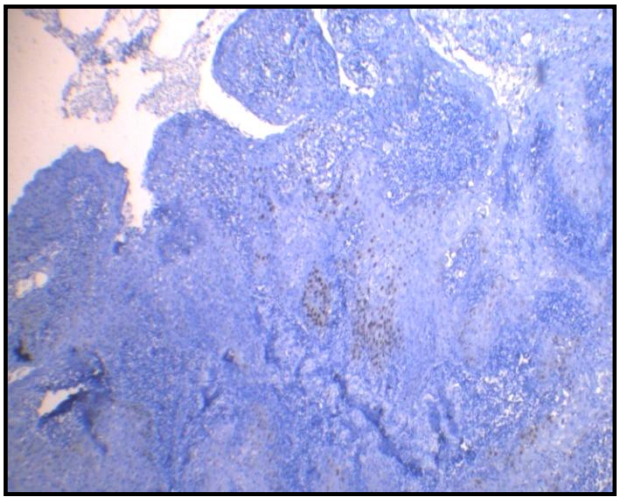


FIGURE 15: Cyclin D1 expression in MDSCC with less than 25% of area of staining (score 1) at 4 X magnification

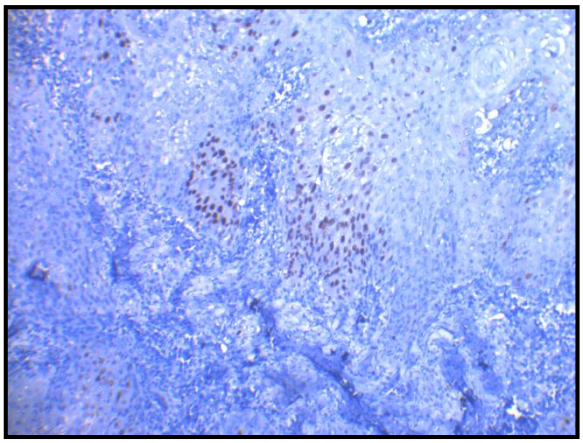


FIGURE 16: Cyclin D1 expression in MDSCC with moderate staining (score 2) at 10 X magnification

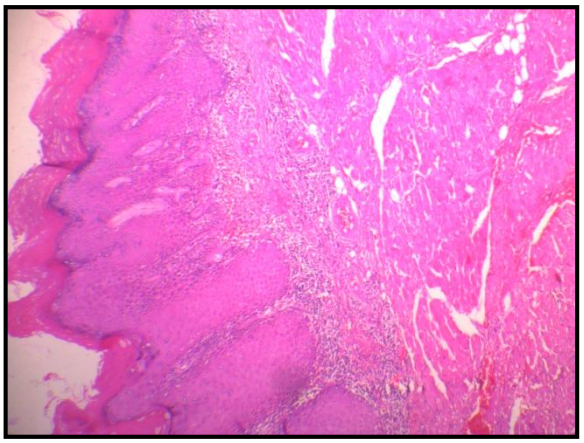


FIGURE 17: Photomicrograph showing H & E stained section of VC at 10 X magnification

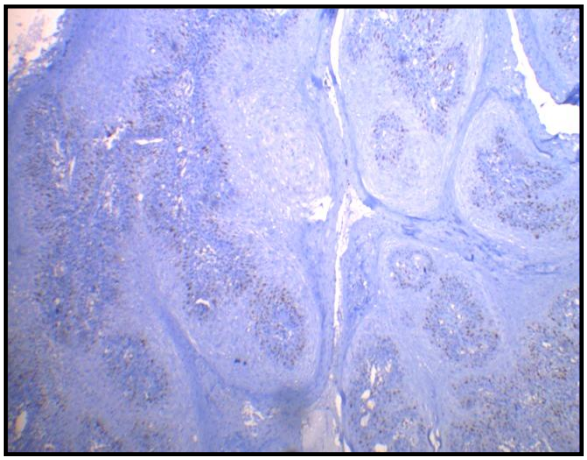


FIGURE 18: Cyclin D1 expression in VC with 50- 74% of area of staining (score 3) at 4 X magnification

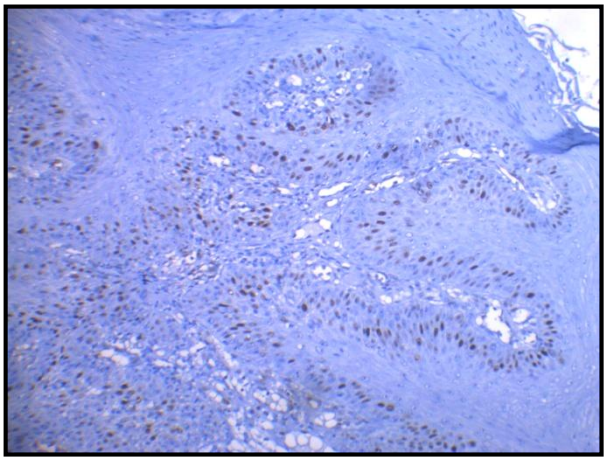


FIGURE 19: Cyclin D1 expression in VC with mild staining (score 1) at 10 X magnification

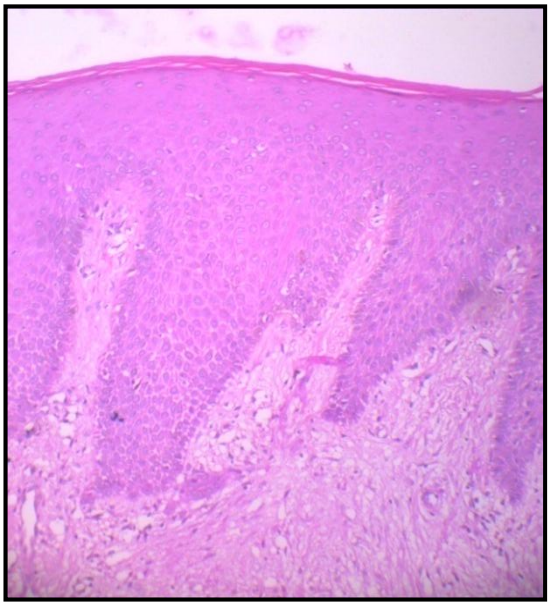


FIGURE 20: Photomicrograph showing H & E stained section of NM at 10 X magnification

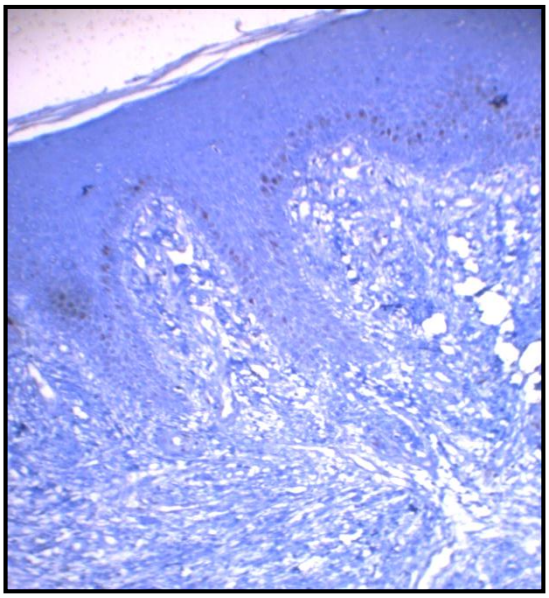


FIGURE 21: Cyclin D1 expression in NM with mild staining (score 1) at 10 X magnification

IMAGES - MCM 2

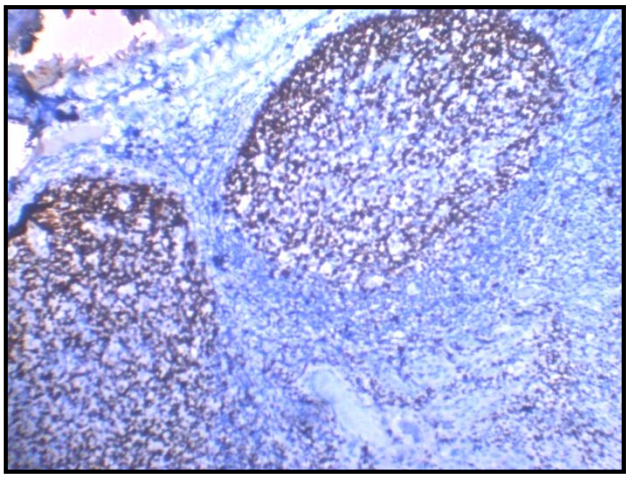


FIGURE 22: MCM 2 expression in positive control tonsil at 10 X magnification

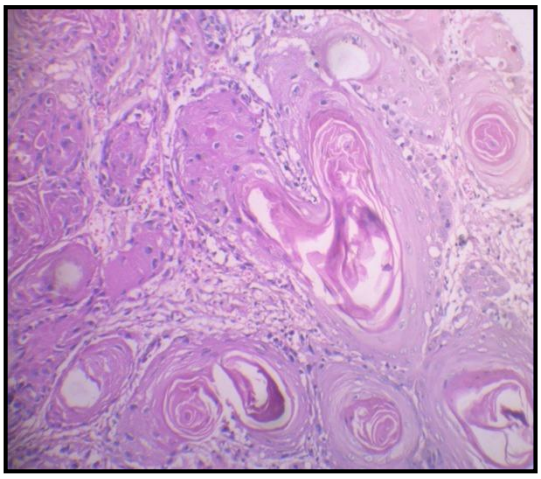


FIGURE 23: Photomicrograph showing H & E stained section of WDSCC at 10 X magnification

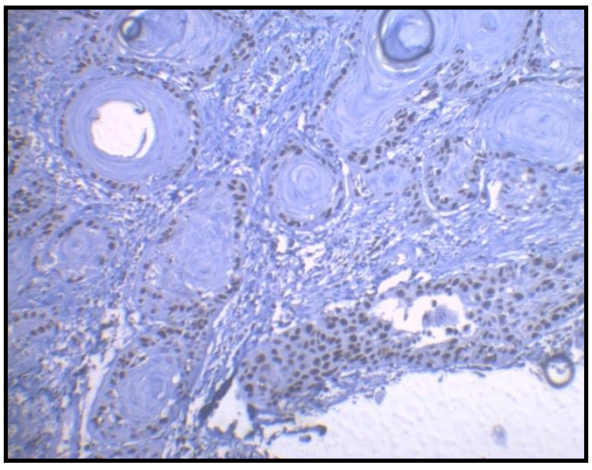


FIGURE 24: MCM2 expression in WDSCC with more than 75 % of area of staining (score 4) at 10 X magnification

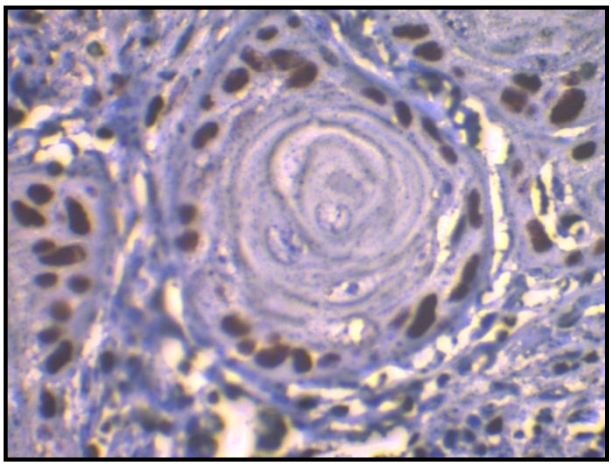


FIGURE 25: MCM2 expression in WDSCC intense staining (score 3) at 40 X magnification

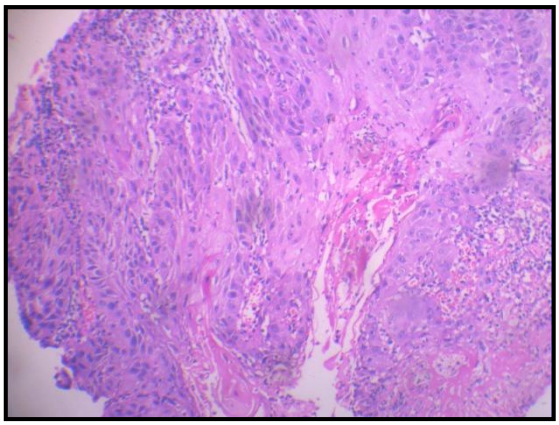


FIGURE 26: Photomicrograph showing H & E stained section of MDSCC at 10 X magnification

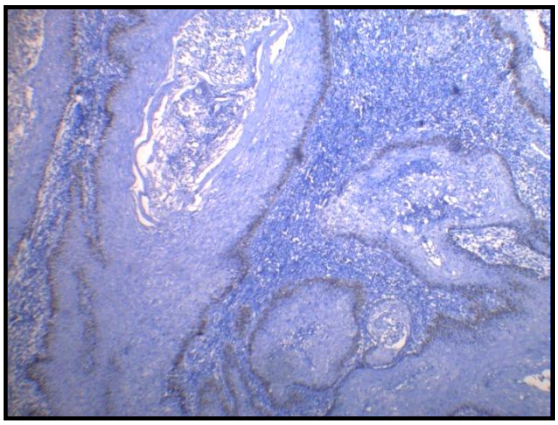


FIGURE 27: MCM2 expression in MDSCC with 50- 74 % of area of staining (score 3) at 10 X magnification

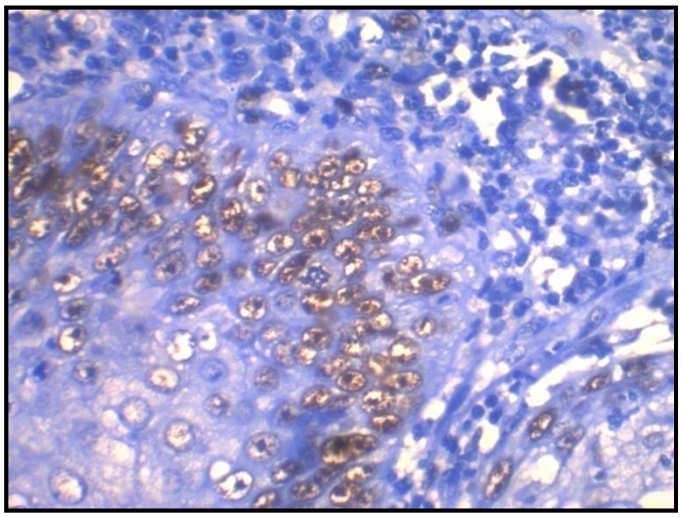


FIGURE 28: MCM2 expression in MDSCC with moderate staining (score 2) at 40 X magnification

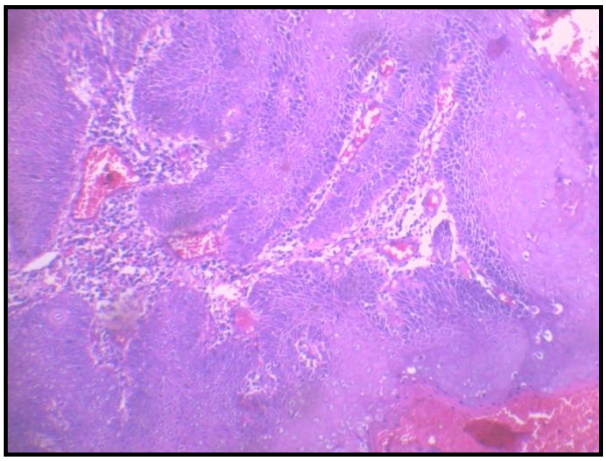


FIGURE 29: Photomicrograph showing H & E stained section of VC at 10 X magnification

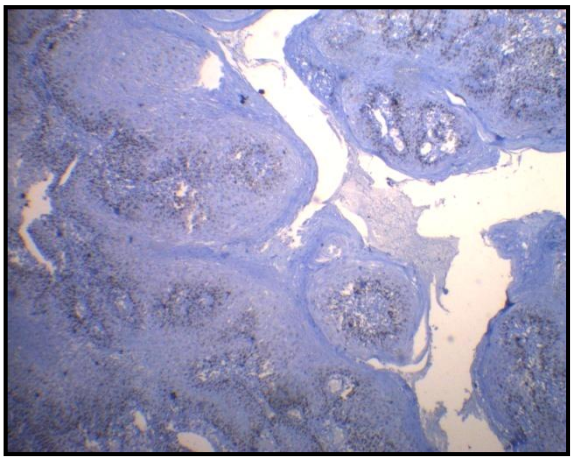


FIGURE 30: MCM2 expression in VC with 50-74% of area of staining (score 3) at 4 X magnification

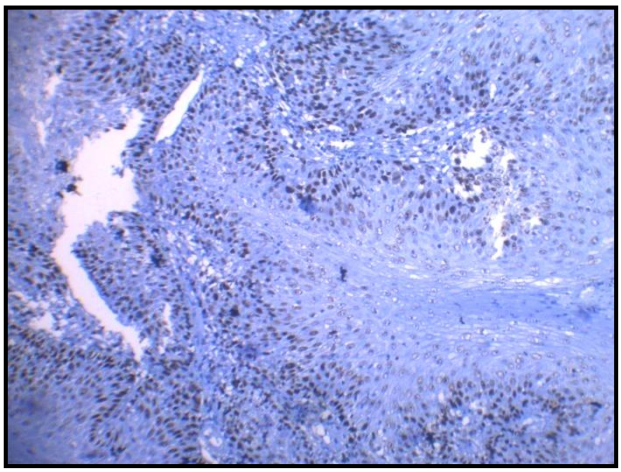


FIGURE 31: MCM2 expression in VC with intense staining (score 3) at 10 X magnification

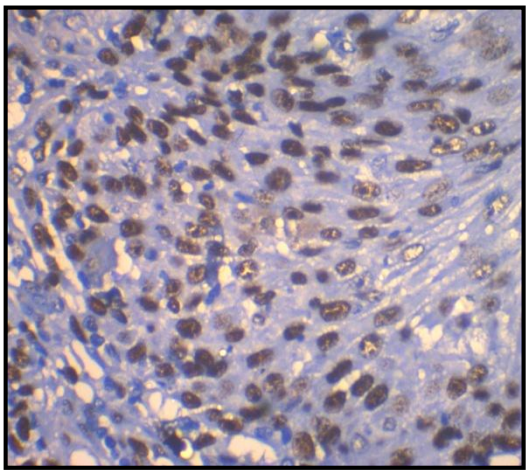


FIGURE 32: MCM2 expression in VC with intense staining (score 3) at 40 X magnification

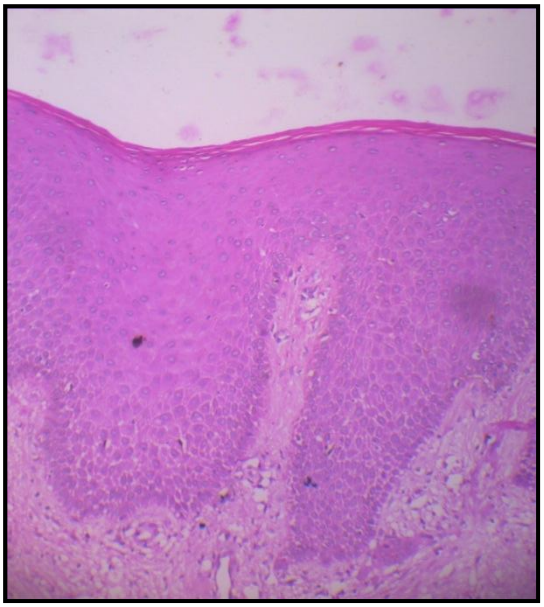


Figure 33: Photomicrograph showing H & E stained section of NM at 10 X magnification

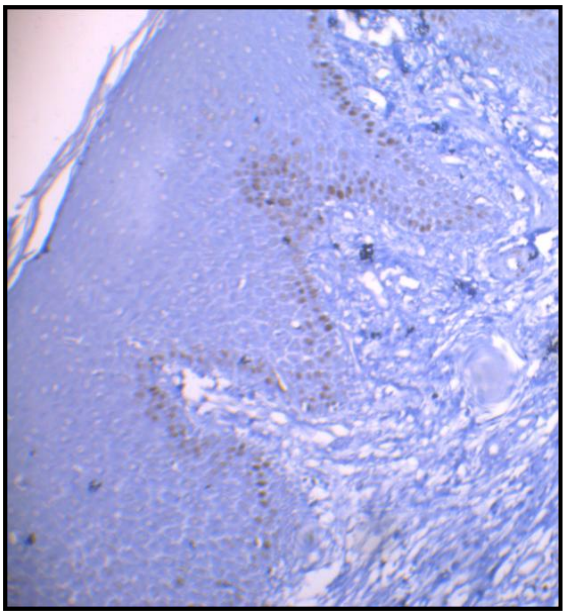


Figure 34: MCM 2 expression in NM with less than 25 % of area of staining (score 1) at 10 X magnification

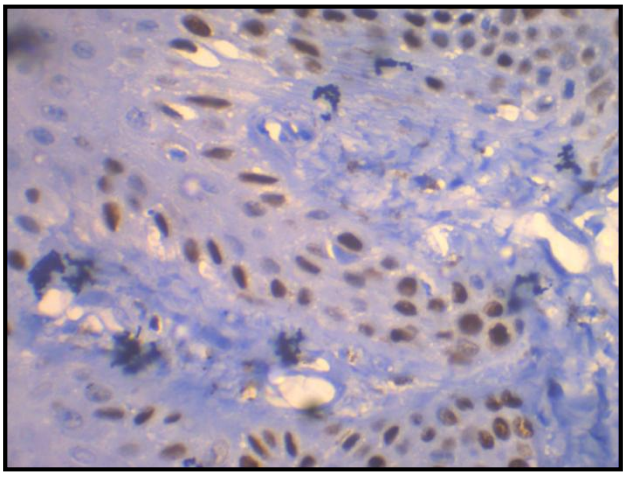


Figure 35: MCM 2 expression in NM with moderate staining (score 2) at 10 X magnification

DISCUSSION

Oral squamous cell carcinoma (OSCC), represents over 90% of malignancies of the oral cavity[1]. Despite the advances in diagnosis and therapy, OSCC continues to have a shorter survival rate. Verrucous carcinoma of the oral cavity, a low grade variant of OSCC is considered a separate clinicopathologic entity due to its unique biologic behavior and limited propensity to metastasize[4]. Tobacco is a major causative factor for OSCC and VC, and for a better understanding, it is of utmost importance to have an in-depth knowledge of the process of carcinogenesis in both these lesions. Carcinogenesis occurs due to genetic alterations, paramount among them are those genes involved in cell proliferation. The study of cell proliferation is important for assessing the tumor behaviour, prognosis and patient survival[11].

Numerous proliferation markers have been developed to detect and quantify proliferation of cells in OSCC[11]. Since cyclin D1 and MCM2 proteins act by stimulating the cell cycle, few studies have been carried out in order to determine whether alterations in expression of these biomarkers are related to carcinogenesis. As literature search did not reveal sufficient studies of immunohistochemical expression of Cyclin D1 and MCM2 in OSCC and VC, the present study has been done to evaluate the expression of these two cell proliferation biomarkers (Cyclin D1 and MCM2) in Oral Squamous cell carcinoma,

and Verrucous carcinoma using commercially available antibodies and paraffin-embedded tissue sections.

In the present study, a total of 80 samples out of which 40 samples of OSCC, 20 samples of VC and 20 samples of normal NM were evaluated for the expression of Cyclin D1 and MCM 2 using standard IHC procedure with anti Cyclin D1 [Rabbit monoclonal antibody – EP12 (PathnSitu Biotechnologies Private Limited)] and anti MCM 2 [Rabbit monoclonal antibody – EP40 (PathnSitu Biotechnologies Private Limited)]

The IHC reactivity was evaluated on the presence or absence of brown coloured end product stain at the site of target antigen. All the cases showed variable intensities of nuclear staining. In the present study, in normal mucosa 60% of the cases showed mild staining intensity of Cyclin D1 in the nucleus of basal cells and few cells in the parabasal layer (Figure 21) which is similar to the study results of **Uma Swaminathan *et al*[45]** and **Angadi and Krishnapillai[17].**

However in the present study 80% of cases showed intense staining for cyclin D1 in WDSCC (Figure 12 and 13) which is similar to the results of **Yuchi Ohnishi *et al*[50]** in which 90% of metastatic foci in WDSCC showed strong staining of cyclin D1. This is in contrast to the study of **Sunit B. Patel *et al*,[52]** **Angadi** and **Krishnapillai[17]** and **Goto *et al*[76]** where mild to moderate intensity of staining was observed in WDSCC.

Similarly, 50% of cases showed intense staining for cyclin D1 in MDSCC (Figure 16) and 50% of cases showed moderate staining for cyclin D1 in MDSCC which is nearly similar to the observations of **Uma Swaminathan *et al*[45].** This is in contrast to the study of **Angadi and Krishnapillai[17],** reported as mild to moderate staining intensity in MDSCC.

40% of cases showed intense staining for cyclin D1 in cases of VC and 30% of cases showed moderate and mild staining (Figure 19) in contrast to **Angadi and Krishnapillai[17]** where predominantly mild staining was observed.

The difference between the mean scores of intensity of staining of cyclin D1 between the study groups was found to be statistically significant (p value = 0.012*)(Table 3)(Graph 1).

The immunoreactivity for area of staining of cyclin D1 in the normal buccal mucosa showed less than 25% of positivity in the nucleus of basal and parabasal cells. The reason for the nuclear staining pattern in our study could be the due to the proliferative activity of the basal layer of cells as cyclin D1 regulates the transition of cells from G1 phase to S phase in cell cycle[25]. When considering the immunoreactivity for area of staining in WDSCC, 80% of samples showed 50- 100 % of positivity (Figure 10 and 11). On analysing the immunoreactivity for area of staining of cyclin D1 in MDSCC and VC 60 % of cases showed less than 25 % of positivity.

The difference between the mean scores of area of staining of cyclin D1 between the study groups was found to be statistically significant (p value = 0.001*)(Table 3) (Graph 1).

In the present study, in WDSCC and MDSCC cyclin D1 was expressed in the outer layers of the epithelial tumour islands and cords since cyclin D1 is an activator of the cell proliferation cycle and peripheral cells are those which are supposed to be the most proliferative and invasive ones in OSCC. In VC, the expression of cyclin D1 was seen mostly in basal and parabasal layers and tends to diminish in the superficial areas[17].

In the present study maximum expression of cyclin D1 in OSCC (WDSCC and MDSCC) was observed which is similar to **Swati Saawarn *et al*[16], Satya N. Das *et al*[18], Masayuki Shiraki *et al* [42], Sunit B. Patel *et al*[52],** and **Angadi** and **Krishnapillai[17].** The higher expression of cyclin D1 observed in OSCC might be explained by the fact that expression of this marker is related to an intense proliferative activity and invasive capacity of the lesions[41,86].

In the present study, the results in normal mucosa showed that in 50% of cases MCM 2 was expressed with moderate intensity of staining (Figure 35).

In our study 100% of WDSCC (Figure 25), 80% of MDSCC and 70% of VC (Figure 32) showed intense staining for MCM 2. These findings are in accordance with **Kodani *et al*[54], Chatrath *et al*[88], Heba N. Shalash[34], and Torres-Rendon *et al*[28].** The difference between the mean scores of intensity of staining of MCM 2 between the study groups was found to be statistically significant (p value = 0.015*) (Table 6) (Graph 2).

In the present study, MCM-2 immunoexpression in epithelium of normal mucosa was seen restricted to the basal and parabasal compartments with few reactive cells in the middle third and totally negative immunoexpression in the upper third. The immunoreactivity for area of staining of MCM 2 in normal mucosa was predominantly less than 50% of expression in basal and parabasal cells. This shows that controlled cell division and proliferation ability occur mainly in basal and parabasal compartment while the superficial cells have lost their proliferative ability. This result was similar to that of **Chatrath *et al*[77], and Chong-Jin *et al*[78].** In contrast, **Torres-Rendon *et al*** [28] investigated MCM-2 expression in normal mucosa and found that MCM2 was mainly expressed at the suprabasal compartment only.

In the current study 70% of the WDSCC showed more than 75% positivity of area of staining of MCM-2 (Figure 24), with expression along the periphery of the invaded epithelial islands, and at the invasive fronts. On the other hand, the central cores of the cell nests

mostly showed negative MCM-2 reaction. These observations are in accordance with **Heba N. Shalash[34], Szelachowska *et al*[56], Scott *et al*[55]** and **Gouvea *et al*[57].** The increase in MCM-2 expression in the peripheral tumor cells and at the invasive fronts suggest a high rate of cellular proliferation and subsequent invasion into the surrounding structures[63].

In MDSCC, 50% of the samples showed more than 75% of positivity for area of staining (Figure 27) evident in the peripheral portions of the invading islands and showed negative in the central core of keratin pearls. These findings are in accordance with **Kodani *et al*[54], Chatrath *et al*[77], Heba N. Shalash[34], and Torres-Rendon *et al*[28].**

In our study 50% of VC cases showed more than 60% of positivity (Figure 30) and 50% of VC samples showed less than 50% positivity for area of staining of MCM2. As mentioned by **Gimenez Conti *et al*[79]**, VC is characterized by a differentiation of a high order and epithelium shows little mitotic activity, hence the cells taking up MCM 2 are lesser.

The difference between the mean scores of area of staining of MCM 2 between the study groups was found to be statistically significant (p value = 0.003*) (Table 6) (Graph 2).

In our study the mean LI of cyclin D1 in normal mucosa was 8.42 ±5.10. The mean LI of cyclin D1 for OSCC (WDSCC and MDSCC) was 20.086 ± 6.840 which is nearly similar to that of **Uma Swaminathan *et al*[45]** where the labelling index of cyclin D1 was 18.88±13.70 in OSCC.

The mean LI of cyclin D1 in VC in our study was 21.68 ±10.90 but the results could not be compared directly due to lack of published reports.

On Comparing the mean Labelling Index (LI) of Cyclin D1 between the normal mucosa, OSCC and VC, statistically significant value (p value= 0.001*) was obtained (Table 8).

In the current study the mean value of LI of MCM 2 in normal mucosa was 18.33± 3.99 similar to observations by **Kodani *et al*[54]** .

The mean LI of MCM 2 for OSCC in the present study was 43.599 ±15.33 which is similar to the value of **Kodani *et al*[54]** but in contrast to the values reported by **Niranjan *et al*[63]., Torres–Rendon *et al*[28]., and Razavi *et al*[36].**

The mean LI of MCM 2 for VC in the present study was 40.67 ± 19.84. This finding is in contrast to the value reported by **Niranjan *et al*[63]** (89.22± 5.51).

On comparing the mean LI of MCM 2 between the normal mucosa, OSCC and VC statistically significant value was obtained (p value =0.002*) (Table 9).

In our study, on comparison of mean LI of Cyclin D1 and MCM 2 in NM, OSCC and VC statistically significant results ($p<0.05$) were obtained in all the study groups (Table 10) (Graph 4). The mean LI of MCM 2 in the study groups was found to be higher than the mean LI of Cyclin D1 in the present study. This could be because MCM-2 proteins identify both cycling cells and non-cycling cells with proliferative potential throughout the cell cycle and expressed in the cell nucleus from early G1 phase.[36]

The present study is probably an early initiative to evaluate the immunohistochemical expression of cyclin D1 and MCM 2 in OSCC and VC. There was a substantial increase in the immunoexpression and mean LI of Mcm-2 and cyclin D1 from Normal mucosa to OSCC.

A similar progressive increase in the immunoexpression and mean LI of MCM 2 and cyclin D1 was observed from Normal mucosa to verrucous carcinoma.

A thorough literature search was done to find out the expression of Cyclin D1 and MCM 2 in verruous carcinoma like lesions. But only very few articles were obtained for reference. Hence the present study can have a place as one of the early studies attempted in expression of MCM2 and Cyclin D1 in VC.

SUMMARY AND CONCLUSION

The aim of the study was to evaluate the immunohistochemical expression of MCM2 and Cyclin D1 in oral squamous cell carcinoma and oral verrucous carcinoma. A total of 20 samples of OSCC, 10 samples of VC & 10 samples of normal mucosa were included in the study and the tissue samples were taken from the archival retrieved from the formalin fixed, paraffin embedded tissues. Immunoexpression of Cyclin D1 and MCM 2 were studied by qualitative and quantitative analysis. Qualitative analysis was done by evaluation of intensity of staining and area of staining. Quantitative analysis was done by calculating the percentage of positively stained cells and determining the Labelling Index (LI).

From the present study done with Cyclin D1 and MCM 2, following conclusions were drawn:

- On evaluating and comparing Cyclin D1 intensity and area of staining between the groups, statistically significant values ($p<0.05$) were obtained.
- On evaluating and comparing MCM 2 intensity and area of staining between the groups, statistically significant values ($p<0.05$) were obtained.
- On Comparison of LI of Cyclin D1 between the groups, statistically significant value ($p<0.05$) was obtained.

- On Comparison of LI of MCM 2 between the groups, statistically significant value ($p<0.05$) was obtained.
- Comparison of LI of Cyclin D1 and MCM 2 in normal mucosa, oral squamous cell carcinoma and verrucous carcinoma statistically significant results ($p<0.05$) were obtained in all the study groups.
- On comparison of mean LI of MCM2 and Cyclin D1, the mean LI of MCM 2 was found to be significantly higher. This can be because MCM 2 proteins are expressed in cell nucleus from early G1 phase. So MCM2 can serve as a more potential biomarker for cell proliferation in OSCC and VC than when compared to Cyclin D1.
- Further studies need to be performed with larger sample size to validate the present findings.

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ANNEXURE I

Omsakthi
ADHIPARASAKTHI
DENTAL COLLEGE & HOSPITAL
Recognised by Dental Council of India
Affiliated to The Tamilnadu Dr.M.G.R Medical University
A Unit of Adhiparasakthi Charitable, Medical, Educational & Cultural Trust

This Ethical Committee has undergone the research Protocol submitted by Dr. Menaka T.R, Post Graduate Student, Department of Oral Pathology & Microbiology, under the title "Immunohistochemical Expression and Evaluation of MCM-2 and Cyclin D1 in Oral Squamous Cell Carcinoma and Verrucous Carcinoma" Ref no : 2016-MDS-BrVI-DEV-13/APDCH under the guidance of Dr.S.Shamala for consideration of approval to proceed with the study.

This Committee has discussed about the Material being involved with the study, the Qualification of the investigator, the present norms and recommendations from the Clinical Research Scientific body and comes to a conclusion that this Research protocol fulfils the Specific requirements and the Committee authorizes the proposal.

Principal

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ANNEXURE II

1.STUDY DATA SHOWING STAINING INTENSITY AND AREA OF STAINING OF CYCLIN D1 AND MCM2

S.NO	HP NO	CYCLIN D1		MCM2	
		Intensity	Area	Intensity	Area
NORMAL MUCOSA					
1	2890/16	2	1	3	2
2	2902/16	2	1	2	1
3	2926/16	1	1	2	1
4	3473/17	1	1	1	1
5	3768/17	3	3	3	2
6	3492/17	2	2	3	2
7	3476	1	1	2	2
8	3217	1	1	1	1
9	3106	1	2	2	2
10	3406	1	2	2	3
WELL DIFFERENTIATED SCC					
1	1367/16	2	3	3	2
2	2128 A/16	3	4	3	4
3	2832 B/16	3	3	3	4
4	3000 C/16	3	4	3	4
5	3138 A/16	3	4	3	4
6	3782 A/17	3	4	3	4

7	3847 A/18	3	2	3	3
8	3866 A/18	3	3	3	4
9	3889 A2/18	2	2	3	3
10	3931/18	3	3	3	4
MODERATELY DIFFERENTIATED SCC					
1	1393/13	3	1	3	1
2	1813 A	2	1	3	4
3	2762 A/16	1	1	1	2
4	2785/16	0	0	1	1
5	3018/16	2	1	3	3
6	3255/17	1	1	3	4
7	3712/17	3	3	3	4
8	3772 /17	3	2	3	4
9	4016 A/18	3	4	3	4
10	3841 A/18	3	1	3	2

	VERRUCOUS CARCINOMA				
S.NO	HP NO	CYCLIN D1		MCM2	
		Intensity	Area	Intensity	Area
1	84 B	2	2	3	4
2	185	2	1	3	4
3	442	1	1	3	2
4	1273 B/13	3	3	3	3
5	1554 B/14	3	2	1	2
6	2036 /15	1	1	2	1
7	2315 B	2	3	3	3
8	3921 A2	3	1	3	1
9	3923 A2	1	1	3	3
10	3934 L	3	1	2	1

2.STUDY DATA SHOWING LABELLING INDEX (LI) OF CYCLIN D1 AND MCM2

S.NO	HP NO	CYCLIN D1	MCM2
		LI	LI
NORMAL MUCOSA			
1	2890/16	2.477%	21.497%
2	2902/16	4.858%	17.391%
3	2926/16	5.4498%	12.572%
4	3473/17	6.425%	13.839%
5	3768/17	13.138%	22.35%
6	3492/17	17.028%	20.795%
7	3476	4.287%	21.35%
8	3217	5.102%	18.085%
9	3106	15.19%	12.857%
10	3406	10.132%	22.558%
WELL DIFFERENTIATED SCC			
1	1367/16	17.468%	28.337%
2	2128 A/16	27.505%	44.311%
3	2832 B/16	13.537%	49.13%
4	3000 C/16	24.457%	48.77%
5	3138 A/16	18.518%	43.816%
6	3782 A/17	26.213%	40.203%
7	3847 A/18	11.787%	29.687%

8	3866 A/18	23.40%	69.655%
9	3889 A2/18	22.357%	45.060%
10	3931/18	25.215%	36.34%
MODERATELY DIFFERENTIATED SCC			
1	1393/13	18.660%	73.113%
2	1813 A	18.201%	54.883%
3	2762 A/16	30%	32.648%
4	2785/16	0	15.384%
5	3018/16	24.449%	46.803%
6	3255/17	15.990%	32.380%
7	3712/17	26.039%	67.012%
8	3772 /17	23.970%	39.583%
9	4016 A/18	17.535%	53.936%
10	3841 A/18	16.397%	20.930%

	VERRUCOUS CARCINOMA		
S.NO	HP NO	CYCLIN D1	MCM2
		LI	LI
1	84 B	32.05%	37.5%
2	185	19.315%	45.858%
3	442	13.469%	64.427%
4	1273 B/13	44.473%	60.199%
5	1554 B/14	28.993%	23.577%
6	2036 /15	14.223%	13.494%
7	2315 B	25.31%	36.346%
8	3921 A2	15.469%	57.110%
9	3923 A2	14.188%	58.071%
10	3934 L	9.318%	10.080%

ANNEXURE III

URKUND

Urkund Analysis Result

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